

Immunity to Toxoplasma gondii:

Studies with sheep and mice

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SUMMARY

Toxoplasma is a major cause of ovine abortion in Great Britain and as yet there is no effective means of control. The work described in this thesis aimed to extend the knowledge of the ovine immune response to infection with Toxoplasma gondii.

It was shown that 2000 M1 strain Toxoplasma oocysts administered orally before pregnancy induced total immunity against a subsequent challenge with 10,000 oocysts at mid gestation. Oocysts which had been treated with 5 or 10 krad of irradiation were also capable of inducing protective immunity although they were still pathogenic.

Specific IgG antibody detected, by ELISA, as early as seven days after infection was found to be directed against certain Toxoplasma antigens by western blotting. It also had opsonising activity in vitro which enhanced phagocytosis of killed parasites by ovine mammary gland macrophages. In addition efferent lymph, collected following infection with Toxoplasma, contained a large number of blast cells, a considerable proportion of which were Ig negative and therefore presumed to be T cells.

The results obtained in this study confirm that protective immunity to Toxoplasma can be induced in sheep. I propose that this immunity is dependent on the priming of T cells which stimulate B cells to secrete specific anti-toxoplasma antibody. Both this antibody and soluble factors released by the primed T cells activate the macrophage population thereby increasing their phagocytic and killing capacities.

DEDICATION

To my parents who have supported and
encouraged me throughout all my studies.

DECLARATION

I declare that this thesis has been composed entirely by myself, and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

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ABBREVIATIONS

ANA	Antinuclear antibodies
B cell	Bone marrow derived lymphocyte
BCGF	B cell growth factor
BD	Border disease
BSA	Bovine serum albumin
CF	Complement fixation
CFA	Complete Freund's adjuvant
DTH	Delayed type hypersensitivity
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
F(ab)2	Antigen binding fragment
FCS	Foetal calf serum
H + E	Haematoxylin + eosin
HBSS	Hanks balanced salt solution
HRP	Horse radish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect haemagglutination
IL-1	Interleukin - 1
IL-2	Interleukin - 2
i.p.	Intraperitoneal
i.v.	Intravenous
kD	Kilo-dalton
Krad	Kilorad
LPS	Lipopolysaccharide
MAF	Macrophage activation factor

MRI	Moredun Research Institute
NK	Natural killer
OD	Optical density
OPD	Ortho phenyl diamine
PAGE	Polyac ^y lamide gel electrophoresis
PBS	Phosphate buffered saline
PEN/STREP	Penicillin and Streptomycin
P	Probability
RF	Rhem ^u atoid factor
RIA	Radioimmunoassay
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
SE	Standard Error
T cell	Thymus derived lymphocyte
T _{DTH}	T lymphocyte responsible for DTH
T _H	Helper T lymphocyte
T _S	Suppressor T lymphocyte
TOXO-GIF	<u>Toxoplasma</u> growth inhibitory factor
V.I. CENTRE	Veterinary Investigation Centre
WB	Wash buffer

LENGTH

cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre

VOLUME

l	litre
ml	millilitre
µl	microlitre

WEIGHT

kg	kilogram
g	gram
mg	milligram
μ g	microgram

CONCENTRATION

M	Molar
mM	Millimolar

MISCELLANEOUS

pH	reciprocal log 10 hydrogen ion concentration
----	--

SYMBOLS

<	less than
>	greater than
=	equal to

CHAPTER 1 Introduction and Literature review

INTRODUCTION

Toxoplasma gondii, a protozoan parasite, was first isolated in 1908 (Nicolle and Manceaux, 1908; Splendore, 1908), however it was not until the early seventies that it was recognised as belonging to the coccidia, following the identification of the sexual phase of its life cycle in cat intestines (Frenkel, Dubey and Miller, 1970; Sheffield and Melton, 1970; Hutchison, Dunachie, Siim and Work, 1970).

The parasite has a worldwide distribution (Frenkel, 1973) and although the sexual phase of the life cycle is extremely host specific, the asexual phase may occur in over 200 species of animal (Levine 1977).

Most Toxoplasma infections in Man and animals are asymptomatic, however it can cause an acute infection with symptoms which, unless treated by chemotherapy can be fatal. After the acute stage of the infection T.gondii enters a chronic phase where it resides in the brain and muscles of the host and this too is normally asymptomatic. If however the host is immunosuppressed either by disease or by drugs, this infection may be reactivated and result in the proliferation of the organism and acute infection. Vertical transmission of infection may lead to severe congenital defects or foetal death in several species including Man and sheep (Wolf and Cowen 1937; Hartley, Jebson and McFarlane 1954). Fortunately in most species congenital transmission will only occur following an acute infection with the parasite.

Toxoplasmosis is of great importance in the sheep industry both in the U.K. and abroad since infection with the parasite during midpregnancy results in abortion and stillbirths. In Tasmania 46

per cent of ovine abortion outbreaks have been attributed to toxoplasmosis (Munday, 1970), in Norway around 20 per cent (Wadeland, 1976a), whereas in the U.K. it is thought to be 18 per cent (Blewett and Watson, 1984). Blewett and Trees estimated that the annual incidence of clinical toxoplasmosis in the U.K. is 1-2 per cent of the breeding flock (Blewett and Trees, 1987). This calculation only includes deaths due to abortion and stillbirths and does not take into account any loss due to barrenness or neonatal mortality, which have also been associated with T.gondii infection (Wadeland, 1977; Huffman, Kirk, Winward and Gorham, 1981). During 1984 there were 13,648,390 breeding ewes in the U.K. (MAFF 1984), therefore if 1-2 per cent were to be infected with T.gondii during mid-pregnancy, one could expect the loss of 140,000-280,000 lambs, assuming one lamb per ewe. The average price per kg of dressed carcase in 1984 was 265p and if one assumes that these lambs would have been of medium weight at the time of selling then the economic loss to British farmers, due to toxoplasmosis, is in the region of 6-12 million pounds per year.

It is important to realise that in addition to T.gondii causing considerable lamb mortality, chronically infected sheep are a potential large reservoir^r for human infection, since the ingestion of meat is a major mode of transmission.

At present there is no practical method of control of ovine toxoplasmosis. Although much has been learned about the nature of T.gondii since the discovery of the sexual phase, the epidemiology is still incompletely understood. Neither chemoprophylaxis or chemotherapy can be effectively used to prevent the clinical symptoms of toxoplasmosis. Furthermore immunisation with killed

T.gondii organisms has also failed to prevent foetal infection in ewes when subsequently challenged during pregnancy (Beverley, Archer, Watson and Fawcett, 1971).

If vaccine development is to be approached in a logical manner then it is important to understand the mechanisms of immunity which the natural infection evokes. At present there is limited information about the ovine immune response to T.gondii, most of the available data involves serological responses.

The main aim of the present study was to extend the knowledge of the ovine immune response to T.gondii infection, to enable in the long term the development of an effective vaccine for ovine toxoplasmosis. Thus it was decided:-

1 To study the humoral immune response following infection with either various doses of intact oocysts or those which had been altered by irradiation.

2 To use the technique of efferent lymphatic cannulation in sheep to study changes in the lymph output after local infection with T.gondii.

3 To investigate whether ovine macrophages have the capacity to phagocytose T.gondii and whether this can be enhanced by specific antibody.

4 To study the antigenicity of the parasite in sheep and to compare this with its antigenicity in mice.

HISTORY

In 1908 Nicolle and Manceaux, whilst studying Leishmania at the Pasteur Institute in Tunis reported a previously unrecognized disease which had occurred in their laboratory gundis. Autopsies revealed an enlarged spleen, congestion of the lungs and organisms resembling babesia were occasionally seen in the blood. Simultaneously, in Brazil Splendore (1908) described a similar disease in rabbits. After further investigation the causative organism was isolated and named Toxoplasma gondii (from the Greek toxon arc, plasma form). This organism was absent from the wild gundi population suggesting that the source of infection was in the laboratory environment (Chatton and Blanc, 1917).

Throughout the 1920's toxoplasmosis remained an obscure disease of laboratory rabbits and guinea pigs, until Wolf and Cowen (1937) reported a case of human congenital infection. Three years later a fatal case was reported in an adult, with lesions and parasites present in several organs including the central nervous system, liver, lymph nodes and the skin (Pinkerton and Weinman, 1940). Natural infections were reported in several other species with symptoms similar to the human cases although the disease in sheep was characterised by a diffuse nonsuppurative encephalomyelitis (Olafson and Monlux, 1942).

The ingestion of meat containing tissue cysts had been recognised as a source of infection in both obligatory and facultative carnivores (Weinman and Chandler, 1954; Desmonts, Couvreur, Alison, Baudelot, Gerbeaux and Lelong, 1965). However it did not account for the high levels of T.gondii infection seen in

herbivores. The first real breakthrough in understanding the parasite's life cycle was when Hutchison found that the faeces of cats fed tissue cysts were infectious to mice even one year after it had been excreted (Hutchison, 1965). Initially it was proposed that the Toxoplasma was protected by intestinal nematode ova (Hutchison, 1967), however several workers dewormed cats before feeding them T.gondii tissue cysts and found that their faeces was still infectious to mice (Sheffield and Melton, 1969; Frenkel, Dubey and Miller, 1969). Examination of the intestinal lining of infected newborn kittens revealed coccidian type schizogony, gametogony and oocysts (Frenkel et al, 1970). Further independent studies established that the infectivity was present within an isosporan oocyst (Overdulve, 1970; Sheffield and Melton, 1970; Hutchison et al, 1970). These findings permitted T.gondii to be classified as a coccidian sporozoan in the suborder Eimeriorina.

CLASSIFICATION

PHYLUM:	Protozo
CLASS:	Sporozoa
ORDER:	Coccidia
SUBORDER:	Eimeriorina
GENUS:	Toxoplasma

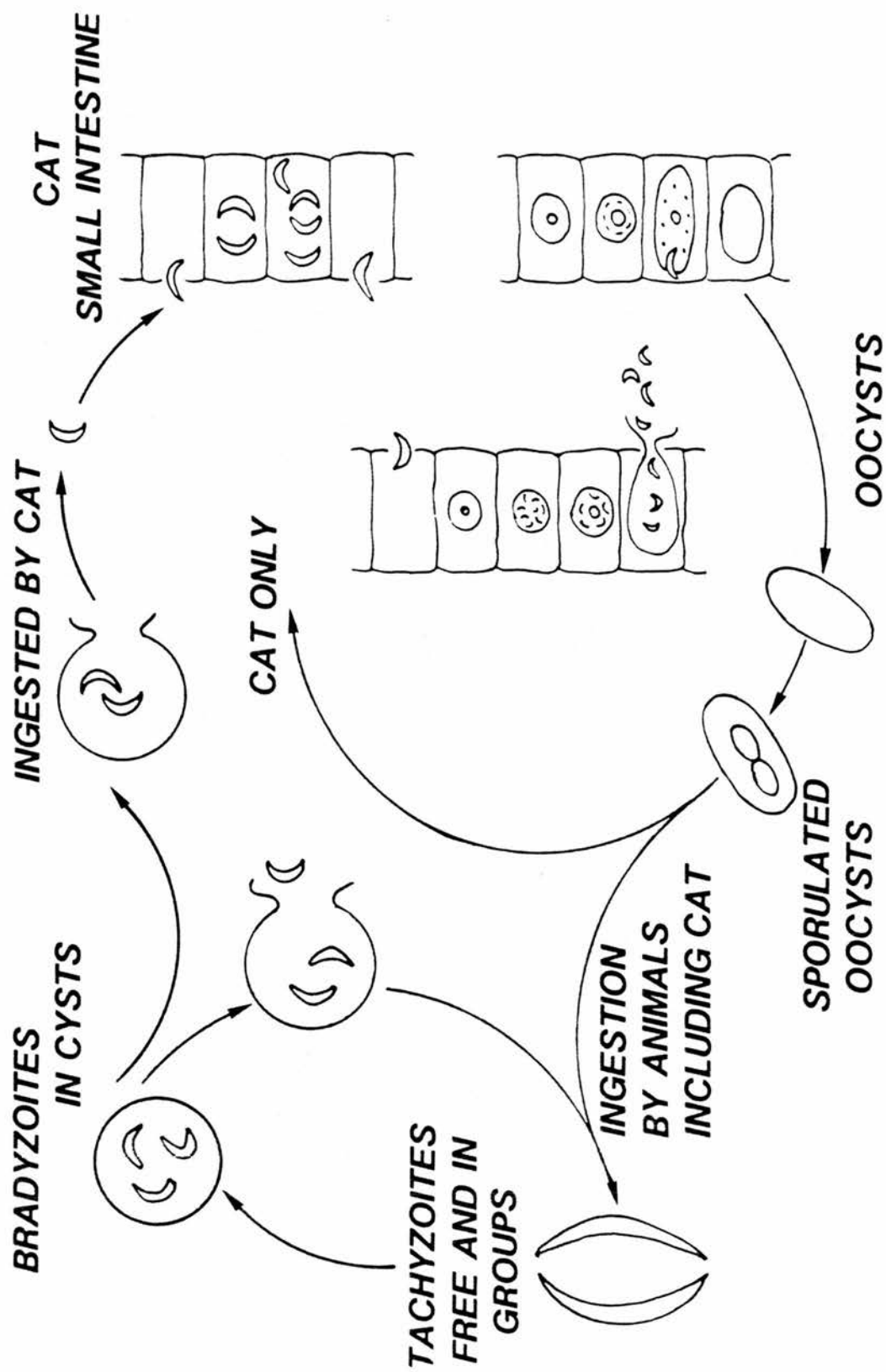
LIFE CYCLE AND STRUCTURE

The complete life cycle of T.gondii has now been elucidated and for ease of explanation it can be divided into an asexual and a sexual phase. Whilst the former can occur in any warm blooded animal the latter only takes place in felids. Figure 1.1 is a diagram^matical representation of the T.gondii life cycle.

ASEXUAL PHASE

The asexual phase of the parasite's life cycle consists of two stages, the tachyzoite and the bradyzoite. The tachyzoite has been studied by both transmission and scanning electron microscopy, it measures approximately $6 \times 2\mu\text{m}$ and is cres^cent shaped, although prior to division it becomes more ovoid (Sheffield and Melton, 1968). The pellicle of the organism consists of two membranes and a row of microtubules. The inner membrane is continuous with thickened areas at both ends (Sheffield and Melton, 1968). The nucleus of the organism is situated in the middle and contains clumps of chromatin whereas the position of the nucleolus is variable. There are many free or membrane associated ribosomes and at least one mitochondrion in the cytoplasm (Sheffield and Melton, 1968). The distribution of the organelles and the morphology of the tachyzoite permit the definition of an anterior and posterior end. Within the anterior end the conoid is situated, this is a truncated cone shaped organelle formed by spirally arranged microtubules. Rhoptries, club shaped structures and micronemes are also present (Gavin, Wanko and Jacobs, 1962). Evidence exists that the conoid and rhoptries are involved in cell penetration (Chiappino, Nichols, and O'Connor, 1984).

FIGURE 1.1 Diagram^m~~atic~~ representation of T. gondii
lifecycle.



Tachyzoites have been shown to be capable of entering any mammalian nucleated cell, irrespective of the cell's phagocytic ability (Werk, 1985) and this is accomplished by active penetration (Aikawa, Komata, Asai and Midorikawa, 1977; Chiappino, et al, 1984). First the apical pole of the parasite is extended and the conoid protruded to permit contact with the host cell, then the tachyzoite induces an aperture 1.5µm wide in the host cell membrane which is the opening of the invagination (Michel, Schupp, Raether and Bierther, 1980). The parasite enters by counter clockwise torsion of the anterior end. A cytoplasmic protrusion from the host cell has occasionally been observed which extended to the apical pole of the parasite forming a plasma bridge (Aikawa et al, 1977). A parasitophorous^o vacuole forms around the tip of the invading parasite, and the host cells shows cytoplasmic projections. Tachyzoites can enter cells within 15-30 seconds which is faster than phagocytosis. However phagocytosis by macrophages has been shown to occur, but this usually follows contact of the host cell with the lateral or posterior part of the parasite (Aikawa et al, 1977).

Although no direct proof has been presented, the available evidence makes it likely that rhoptries secrete a proteinaceous material which aids the organism's entry into the cell (Schupp, Michel, Raether, Niemeitz and Uphoff, 1978). Norrby and Lycke (1967) isolated a protein of molecular weight 70 kD from tachyzoites. This protein, named penetration enhancing factor (PEF) helps invasion even when present at very low concentrations, however it does not increase phagocytosis as determined by intake of latex particles (Lycke, Carlberg and Norrby, 1975) and

furthermore after invasion has occurred the amount of extractable PEF decreases. Cationic polypeptides have also been shown to increase active penetration (Werk, Dunker and Fischer, 1984) however the relationship between these and PEF has not been established.

Once inside the cell, live parasites multiply within the parasitophorous vacuole by a process of endodyogeny, a specialised form of division in which two daughter cells form within the mother cell which eventually ruptures to release the new cells (Gavin et al, 1962; Sheffield and Melton, 1968). Even within the phagosome of macrophages multiplication occurs unhindered since the tachyzoite can prevent both the acidification of the phagosome and the fusion of it with the lysosomes which thereby inhibits intracellular killing (Jones, Yeh and Hirsch, 1972; Sibley, Weidner and Krahenbuhl, 1985). The tachyzoite continues to multiply within the vacuole until the cell ruptures releasing the parasites into the circulation. This parasitaemia enables the tachyzoite to infect any part of the host's body.

At some stage, the fast multiplying tachyzoites transform into bradyzoites which divide more slowly (Dubey and Frenkel, 1976). The factors which lead to this change are not well understood, however it is unlikely that development of the host's immunity is the only criteria since bradyzoites have been detected as soon as 3-4 days after infection with tachyzoites (Dubey and Frenkel, 1976). Bradyzoites are very similar in structure to tachyzoites, however they are slightly smaller, contain several glycogen granules which stain with periodic acid-Schiff and have a nucleus situated towards the posterior end. The bradyzoites are contained

within a tissue cyst, the outer layer of which is almost certainly a trilaminar host derived membrane which originates from the parasitophorous vacuole (Wanko, Jacobs and Gavin, 1962; Jones, Bienz and Erb, 1986). Immunohistological methods have revealed that the tissue cyst wall also contains parasite antigens (Conley and Jenkins, 1981). Presumably the host constituents are present in sufficient quantity to prevent the onset of an immune response. The membrane has also been shown to be an active structure which can survive even after the host cell has disappeared. It may play a role in the nutrient transport for the bradyzoites (Jones et al, 1986). The cyst wall is lined with an amorphous material which also fills the spaces between the bradyzoites (Wanko et al, 1962).

Bradyzoites also multiply by endodyogeny within the tissue cyst (Wanko et al, 1962; Conley and Jenkins, 1981) which ruptures releasing bradyzoites which can sometimes evoke an inflammatory reaction (Conley and Jenkins, 1981).

Tissue cysts are reported to be found more consistently in the host's brain and muscles than elsewhere in the body. A serial kill of infected sheep demonstrated that although cysts were present in numerous organs at 64 days post inoculation, by day 119 they could only be isolated from brain, muscle and intestine (Dubey and Sharma, 1980). The reasons for this predilection have not been established.

SEXUAL PHASE

After a cat ingests T.gondii the parasite is able to undergo both the sexual and asexual phases of its life cycle. Dubey and Frenkel (1972) studied in detail the cycle which takes place in the

cat's small intestine following ingestion of bradyzoites within tissue cysts, which eventually gives rise to oocysts.

The tissue cyst wall is dissolved by the proteolytic enzymes present in the stomach and small intestine, the released bradyzoites then penetrate the epithelial cells of the small intestine and go through five morphologically distinct stages designated Type A -E. Gametes appear to arise from either Type D or E. The female gamete, the macrogamete, is spherical in shape and contains several cell organelles, whereas the male microgamete consists mainly of nuclear material and has two flagella. Fertilisation occurs when the microgamete penetrates the macrogamete, this takes place within the cell. However from the available evidence it is unclear whether micro- and macrogametes develop in the same or different cells. If the latter is true presumably the microgamete is able to penetrate other cells in order to reach and fertilise the macrogamete. There is little cytoplasmic change in the macrogamete once fertilisation has occurred however a five layer thick wall forms around the gamete (Ferguson, Hutchison and Siim, 1975).

Oocysts are released into the intestinal lumen following rupture of the epithelial cells, they measure 10 by 12 μm in diameter. Sporulation of the oocyst occurs outside the felid one to three days after excretion depending on environmental conditions. Within each oocyst two sporocysts form, these are ellisoidal (6 by 8 μm) and have a smooth surface consisting of four plates. Four sporozoites are present in each sporocyst, these gain access to the outside through the joins that exist in the sporocyst wall, and then by the rupture of the oocyst wall at one or more

points. Ultrastructurally sporozoites resemble tachyzoites, however they lack both micronemes and rhoptries (Sheffield and Melton, 1970).

If cats ingest bradyzoites, almost 100 per cent of them will shed oocysts in their faeces within 3-10 days after infection. However less than 50 per cent of cats will excrete oocysts if they are fed with either tachyzoites or sporozoites within oocysts. Furthermore the prepatent period, that is, the time from ingestion to shedding is more than 19 days (Dubey and Frenkel, 1976). This data indicates that carnivorism is important in the production of oocysts by cats.

RESISTANCE OF THE PARASITE TO ENVIRONMENTAL CONDITIONS

The different stages in the T.gondii life cycle show varying resistance to environmental conditions. Understanding of these properties is important in both epidemiological studies and in the handling of the organism.

Tachyzoites are killed by drying or immersion in either tap water, 70 per cent ethanol or the common detergents (Frenkel, 1973). They are able survive^{to} in a one per cent trypsin solution for up to three hours, but are dead within minutes if exposed to pepsin (Jacobs, Remington and Melton, 1960; Sharma and Dubey, 1981).

Distilled water will kill bradyzoites within 30 minutes, whereas they will survive for considerably longer if saline is added (Frenkel, 1973). Bradyzoites will survive in one per cent pepsin for up to two hours with no difference in viability, whereas

incubation in one per cent trypsin for the same time decreases the infectivity 100 fold (Jacobs et al, 1960; Sharma and Dubey, 1981). Heating to 56°C for 10-15 minutes will kill all the bradyzoites (Work, 1968) and freeze thawing will kill at least a proportion of them (Work, 1968; Dubey, 1976).

Oocysts can survive in cat faeces or in tap water for considerably more than a year, and at a wide range of temperatures. (Hutchison, 1967; Frenkel, Ruiz and Chinchilla, 1975). They can be stored in two per cent sulphuric acid for their lifespan (Dubey, 1977). Furthermore they can survive for six hours in 10 per cent formalin and for 24 hours in concentrated sulphuric acid (Frenkel, 1973). However oocysts can be destroyed by treatment with either 10 per cent formalin for 24 hours or 10 per cent ammonium hydroxide for 10 minutes (Dubey, Miller and Frenkel, 1970) or by heating to 60°C for 30 minutes (Dubey et al, 1970). Drying also decreases the viability of the oocysts (Frenkel and Dubey, 1972).

EPIDEMIOLOGY

Most infections with T.gondii are asymptomatic therefore analysis of clinical outbreaks in either man or animals gives an inaccurate picture. Due to this, epidemiological studies rely on parasite isolation and serological evidence. Since the former is often difficult to achieve, either due to the need to kill the animal or difficulties with techniques most studies tend to concentrate on the serological aspects.

Toxoplasma gondii has been isolated from most warm blooded animals (Frenkel, 1973) amongst them the manatee (Buergelt and

Bonde, 1983). Numerous serological surveys for anti-toxoplasma antibody have been carried out in various populations. Data from recent human studies demonstrated that in Nigeria 27 per cent of the population tested had positive titres (Osuyemi, Synge, Agbonlahor and Agbavwe, 1985); 30 per cent in South Australia (Johnson, Roberts and McDonald, 1980); 39 per cent in Brazil (Lovelace, Moraes and Hagerby, 1978); whereas in Borneo, in seven different locations, between 9.7 - 51 per cent of the people had serological evidence of T.gondii infection (Durfee, Cross, Rustam and Susanto, 1976).

Domestic animals have also been tested and the data indicates that between 6-35 per cent of cattle, 7-18 per cent of horses and 14 per cent of goats have anti-toxoplasma antibodies (van Knapen, Franchimont and van der Lugt, 1982; Connor and Hallwell, 1985; Osiyemi et al, 1985; Ugglä and Hjort, 1984). The prevalence of anti-toxoplasma antibody in swine ranges between 0-35 per cent (Moreno, Martinez-Gomez and Hernandez-Rodriguez, 1985; Ugglä and Hjort, 1984; van Knapen et al, 1982). However the prevalence of antibody has decreased in some locations in recent years due to changing management and earlier slaughter (van Knapen et al, 1982).

Numerous serological studies have been performed on the sheep population and the more recent ones indicate that the prevalence of antibody to T.gondii is around 15.2 per cent in Scotland (McColm, Hutchison and Siim, 1981); 46 per cent in Norway (Waldeland, 1976a); 60-66 per cent in Sweden (Ugglä, Beskow, Schwan, Bergquist and Waller, 1983; Ugglä and Hjort, 1984); 22 per cent in Nigeria (Osiyemi et al, 1985) and between 4-51 per cent in the west of the USA (Riemann, Willadsen, Berry, Behymer, Garcia, Franti and Ruppanner, 1977).

Several factors are involved in dictating the occurrence of Toxoplasma infection. The prevalence of antibody in a given population has been shown to increase with age, however the infection rate does eventually reach a plateau (Johnson et al, 1980). Climate also appears to have an affect whereby wetter and warmer areas experience greater rates of infection (Osiyemia et al, 1985; Ugglä et al, 1983), presumably because of the oocyst's susceptibility to drying and low temperatures (Frenkel and Dubey, 1972). In the early literature variations in the prevalence of Toxoplasma infection at different altitudes was reported, with more seroconversion nearer to sea level (Walton, de Arjona and Brenchoff, 1966; Gibson and Coleman, 1958).

In addition to these environmental factors, the modes of transmission of T.gondii play an important role in determining the prevalence of infection.

CONGENITAL TRANSMISSION

Vertical transmission was first observed in human beings in 1937 (Wolf and Cowen) and later studies showed that it could only occur if acute infection took place during pregnancy (Desmonts and Courieur, 1974). Vertical transmission of Toxoplasma in sheep was first observed in 1954 (Hartley et al) and as with human beings, ewes will produce infected offspring only once (Watson and Beverley, 1971). In contrast infected mice can produce successive infected litters and vertical transmission can occur through successive generations (Beverley, 1959).

CARNIVORISM

The idea that Toxoplasma could be spread by ingestion of contaminated meat was first proposed by Weinman and Chandler (1954)

who demonstrated that pigs could be infected by eating flesh containing T.gondii. Since bradyzoites are resistant to proteolytic enzymes, they should be able to survive in the digestive tract for long enough to cause infection. However as previously mentioned bradyzoites are killed by heating to 60°C and usually by freezing and thawing (Work, 1968), therefore the danger of transmission is limited to the ingestion of raw or undercooked meat or in the handling of it (Desmonts et al, 1965; Kean, Kimball and Christenson, 1969; Stray-pederson and Lorentzen-Styr, 1980). Serological studies in Paris where raw meat is commonly eaten indicated that 84 per cent of women tested during pregnancy had positive titres to Toxoplasma (Desmonts and Courieur, 1974) compared to other countries such as Scotland where only 16 per cent were positive (Williams and Williams, 1979).

FAECAL

The investigative work that led to the isolation of T.gondii oocysts from cat faeces (Hutchison, 1965; Hutchison, 1967; Hutchison Dunachie, Siim and Work, 1969; Frenkel et al, 1970; Hutchison et al, 1970; Overdulse, 1970; Sheffield and Melton; 1970) has had a considerable impact on the understanding of transmission of T.gondii especially in herbivores. Serological surveys and also the isolation of the parasite from cat faeces has given various rates of infection in the cat population. Of the cats tested in Costa Rica 60 per cent were positive by serology (Ruiz and Frenkel, 1980); 25.7 per cent in Scotland (McColm et al, 1981) and 55 per cent in Australia (Watson, Farrow and McDonald, 1982). It is apparent that the prevalence of antibody to Toxoplasma in a given cat population increases with age whereas the parasite isolations

from faeces decrease with maturity (Ruiz and Frenkel, 1980; Watson et al, 1982). Some workers have found that infection is related to the onset of hunting by the kitten (Wallace, 1969), however in Costa Rica one month old kittens were found to be shedding oocysts, this coincided with the weaning period, when mothers were still supplying food for their young which was presumably infected with T. gondii (Ruiz and Frenkel, 1980).

Cat free environments enable direct comparisons to be made between the association of cats and the prevalence of Toxoplasma infection. The subjects of one such study were three Pacific islets (Wallace, Marshall and Marshall, 1972). One of these islets was inhabited by several species of animal including man and cats; the second had no humans and the third had neither man or cats. Serological evidence of Toxoplasma was only found on the islets where cats were present (Wallace et al, 1972). Furthermore in New Guinea, between 16-34 per cent of humans had antibody to T.gondii in villages where cats were present whereas cat free villages had less than two per cent seroconversion (Wallace, Zigas and Carleton Gajdusek, 1974).

Other members of the cat family such as jaguarundi and ocelots are able to excrete T.gondii oocysts (Miller, Frenkel and Dubey, 1972; Jewell, Frenkel, Johnson, Reed and Ruiz, 1972) and there are indications that human beings in contact with such animals can become infected with the parasite (Wallace et al, 1974).

INHALATION

Sheep have been infected intranasally with T.gondii (Hartley, 1961) and isolated cases of accidental infection of humans by

inhalation of a spray containing infectious organisms have been reported (Rawal, 1959; Neu, 1967).

VENEREAL

Toxoplasma has been isolated from sheep semen (Spence, Beattie, Faulkener, Henry and Watson, 1978; Teale, Blewett, Miller and Buxton, 1982), but it is unlikely that this plays a significant role in the spread of toxoplasmosis (Blewett, Teale, Miller, Scott and Buxton, 1982). No evidence exists that this mode of transmission occurs in humans, (Price, 1969).

ARTHROPOD

Experimental transmission of T.gondii is possible with *Musca domesticalis* (Wallace, 1973) however this was not observed in natural infections (Frenkel et al, 1975).

MILK


Viable T. gondii organisms have been recovered from the colostrum of cows and pigs (Sanger, Chamberlain, Chamberlain, Cole and Farrell, 1953; Sanger and Cole, 1955). However there was no evidence that ingestion of this milk led to infection.

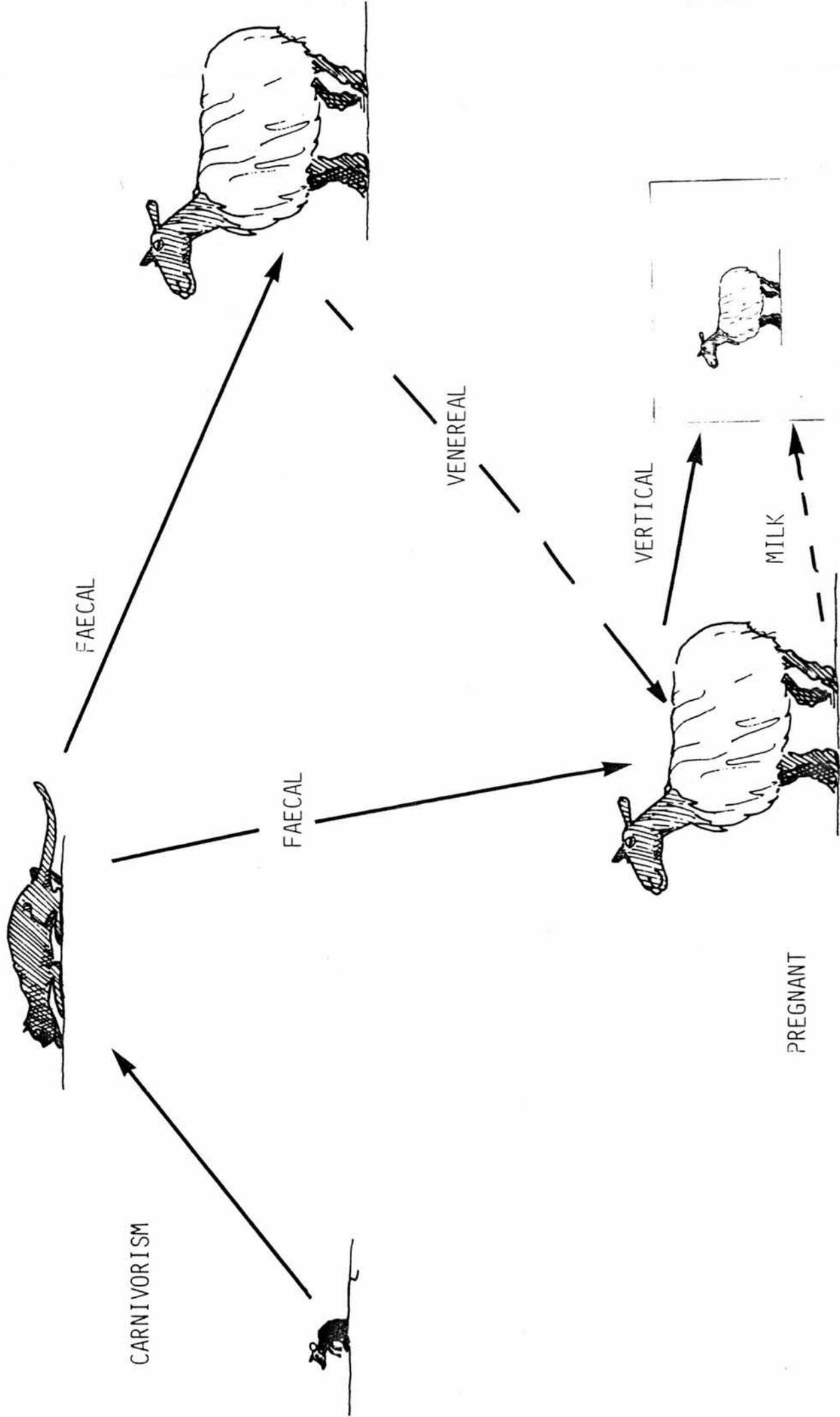
The possible ways of spreading Toxoplasma infection in the sheep population are described in figure 1.2.

SEROLOGY

The diagnosis of toxoplasmosis in most species is dependent on serological techniques. In order to be of use these tests must be both specific and sensitive. High antibody levels can still be detected in serum years after infection, therefore a suitable test

FIGURE 1.2 Epidemiology of T. gondii with respect to
the sheep host.





must be able to distinguish between chronic infection and a recent onset. This can be achieved by detecting a rising titre in sequential samples of serum or by the presence of specific IgM antibody (Remington, Miller and Brownlee, 1968). Epidemiological studies to determine the prevalence of Toxoplasma infection also rely on serological tests.

There is a multitude of serological tests for the detection of anti-toxoplasma antibodies, and this may indicate that no one test is entirely satisfactory.

Serological tests can conveniently be divided into those which use the intact parasite and those which use a fractionated antigen preparation.

INTACT PARASITE

Dye Test

Sabin and Feldman (1948) developed a test which is still widely used today and which is considered by many to be the standard assay. The test relies on the observation that viable tachyzoites takes up methylene blue dye, so that the chromatin stains lightly and the cytoplasm is dark blue. However in the presence of immune serum and a heat liable accessory factor only the chromatin on the tachyzoite stains and this therefore allows the presence of specific antibody to be visualised by light microscopy. From this standardisation and microadaption of the dye test followed (Beverley and Beattie, 1952; Feldman and Lamb, 1966). However although this test has been shown to be both sensitive and specific it has several major disadvantages such as a prozone effect where a 1:2 or a 1:4 dilution of serum can be negative but a

1:8 dilution positive. The test also requires the use of live virulent RH strain tachyzoites and these represent a potential hazard to the user who must be specifically trained in their handling. A source of accessory factor obtained from fresh serum is also necessary for the test.

Fluorescent Antibody Test

Goldman (1957) first used a fluorescein conjugated antiserum in an inhibition assay. The conjugated anti-toxoplasma antiserum was mixed with test serum before incubation with Toxoplasma tachyzoites which had already been dried onto a slide. A decrease in fluorescence compared to a sample with no test serum was indicative of antibody to T.gondii in the test serum. Although specific this test required the conjugation of an anti-toxoplasma antiserum and the result was difficult to read. The development of an indirect immunofluorescent antibody test (IFAT) increased the sensitivity, allowed a titration of the test serum to be performed and separated the antibody response into IgM and IgG isotypes (Fletcher, 1965). Rigorous testing showed that the test was not cross-reactive for other parasites and that it gave comparable titres to the dye test. As with the dye test whole parasites are used, indicating that the antigens on the surface of the parasite are being detected.

Several workers have shown that false negative results can occur in the IgM IFAT test due to saturation binding by specific anti-toxoplasma IgG (Pyndiah, Krech, Price and Wilhelm, 1979; Filice, Yeager and Remington, 1980) but that separation of the IgM fraction by gel filtration before its use in the test can give a high yield of IgM antibody with very little contaminating IgG

(Pyndiah et al, 1979; Filice et al, 1980; Ordonez, Newman and Stone, 1982). This procedure dramatically decreased the number of false negatives whereas removal of IgG by Protein A gave unsatisfactory results. Furthermore performance of this technique was feasible on a routine basis. False positive results could occur due to rheumatoid factor (Pyndrah et al, 1979), or antinuclear antibodies (ANA) present in the test serum. Fortunately removal of IgG by fractionation eliminated these reactions (Filice et al, 1980). Another possible drawback of the IFAT was that polar staining could occur due to a non-specific reaction by polymeric IgM (Franco, Sulzer, Higby and Peralta, 1980), however this could be removed by treatment with 2- mercaptoethanol.

Unfortunately the IFAT is time consuming and open to subjective error. Automation of the procedure was attempted with a commercially available fluometer, called FIAX (Gordon, Duncan and Kingsley, 1981), and when compared with the IFAT 89 per cent of the results were in agreement, however FIAX titres were regularly four fold higher. Unlike the IFAT these results are read objectively but a soluble antigen preparation was used rather than the whole parasites used in the IFAT . It therefore appears that automation of the IFAT is still in the future.

Agglutination Test

In the agglutination test formalised tachyzoites are used, which requires a source of large numbers of organisms. Initial tests gave lower titres than those obtained with either the dye test or the IFAT (Fulton and Turk 1959; Fulton, 1965), but recent developments have shown that joint infection of mice with tachyzoites and sarcoma cells give a greater yield of antigen and

also increase sensitivity thereby producing comparable results to the dye test (Desmonts and Remington, 1980). This test is free from cross reactions with other parasites but as in other tests IgM can bind non-specifically to the organisms to give false positives (Desmonts and Remington, 1980), as previously mentioned treatment with 2-mercaptoethanol eliminates this reaction.

The agglutination test is widely used in France for screening purposes however since it detects predominantly IgG antibodies it is not suitable for diagnosis of infection of recent onset (Desmonts and Remington, 1980). Although the test is very simple to perform it is sensitive to both pH and temperature changes and therefore standardisation is essential.

SOLUBLE ANTIGEN PREPARATIONS

Complement Fixation Test

A complement fixation (CF) assay was almost certainly the first serological test used in the diagnosis of toxoplasmosis (Warren and Sabin, 1942). Further adaptations of the technique have been reported (Sabin, 1949; Fulton and Fulton, 1965), but it is generally believed that the CF antibody titres do not compare well with those of either the dye test or the IFAT (Frenkel 1948; Adams, Kabler, Cooney and Adams, 1949; Camargo, Ferreira, Mineo, Takeguti and Nakahara, 1978). Antibodies detected by the CF test tend to appear transiently and are often indicative of a recent infection.

Indirect Haemagglutination Test

Indirect haemagglutination (IHA) tests operate on the principal that specific antibody will crosslink red blood cells coated with soluble antigen. Initial attempts at the development

of this assay to detect anti-toxoplasma antibody were unsuccessful mainly due to the instability of the sensitized red blood cells (Mitchell and Green, 1960). This difficulty was overcome in part by treatment of cells with pyruvic aldehyde (Jennis, 1966). A further adaptation of this procedure resulted in an antigen preparation which was stable for at least one year (Thorburn and Williams, 1972).

When tested on sera from various patients no positive dye test results were found in the absence of IHA titres. However only 88 per cent of the sera tested had titres within a four fold limit in both tests (Thorburn and Williams, 1972). Furthermore no correlation was found between IFAT and IHA titres (Panigrahi, Mithal, Motiapatra and Shyam Sundun, 1978). Absorption of immune sera with the sensitized cells used in the IHA test had no effect on the titres of the serum in either the dye test or the IFAT (Fleck, 1963). The IHA test detects both IgM and IgG antibodies but titres appear later than the dye test titres possibly due to a cytoplasmic antigen preparation^a being used.

Radioimmunoassay and Enzyme linked immunosorbent assay

Both radioimmunoassays (RIA) and enzyme linked immunosorbent assays (ELISA) work on the principle that soluble antigen can be bound to polystyrene or polyvinyl surfaces and that specific antibody which binds to this antigen can be detected by either a radiolabelled or enzyme linked specific antiserum.

A RIA has been developed which was both specific, sensitive and could detect individual isotypes. However as the half life of the ^{125}I used is relatively short, conjugate frequently needed to be prepared (Finlayson, 1980).

An ELISA was first developed in 1976 (Voller, Bidwell, Barttell, Fleck, Perkins and Oladehin). The coating antigen was prepared by sonication of RH tachyzoites which had been freeze thawed. Cell debris was removed by centrifugation and the supernatant used. This test showed more correlation with the IHA presumably since very similar antigen preparations were used, than the dye test and was unable to discriminateⁱⁿ between sera with medium or high dye test titres. Further studies with a freeze thawed lysate of tachyzoites which included both membrane and cytoplasmic antigens have shown good correlation with IFAT test (Walls, Bullock and English, 1977).

An isotype specific ELISA was produced with an antigen preparation obtained from tachyzoites lysed with distilled water and then sonicated. Although the IgG-ELISA titres were comparable with those of the IgG-IFAT the IgM -ELISA was much more sensitive than the IgM-IFAT (Camargo et al, 1978). Possibly as a result of IHA assay measuring both IgM and IgG, no correlation between titres in this test and either of the ELISA test was found (Camargo et al, 1978).

For diagnostic purposes a system which accurately detects IgM is vital. Since some false positives were detected in the IgM-ELISA a double sandwich technique was developed (Naot and Remington, 1980). Antiserum raised against IgM antibodies is allowed to coat the wells of a microtitre plate, after which the test serum is added and any IgM present is captured by the anti-IgM. Antigen is added and if any specific anti-toxoplasma IgM is present it will bind to it. This bound antigen is then detected with an anti-toxoplasma conjugated antiserum. This assay is very

sensitive, there is no competition from IgG antibodies and most importantly it correlates well with clinical symptoms (Naot and Remington, 1980; Naot, Desmonts and Remington, 1981). Although the presence of RF and ANA together had been shown to cause false positives, this can be avoided by the $F(ab)_2$ fragment of antiserum being used (Naot, Barnett and Remington, 1981).

Adaptation of the ELISA system enables different antigen preparations such as polysaccharide extracts to be used (Mineo, Camargo and Ferreira, 1980). In addition monoclonal antibodies raised against Toxoplasma can be used instead of complete sera (Cesbron, Capron, Orlaque and Santora, 1985).

Although the ELISA is an extremely useful test it has yet to be standardised. For example slight alterations in antigen preparations make significant differences in results and variation can also occur due to differences in polystyrene plates (Denmark and Chessum, 1978).

Other Serological Techniques

Several other techniques have been developed. A latex agglutination test was developed but was not recommended for diagnostic use (Lunde and Jacobs, 1967). The carbon immunoassay is inexpensive and easy to perform, however it is less sensitive than both the dye test and the IFAT (Bergquist and Waller, 1983; Waller, Ugglä, Bergquist and Walter, 1985) and a diffusion in gel ELISA has also been developed recently (Ugglä and Nilsson, 1985).

Several of the tests described above can be automated to some degree (CF, IHA, RIA, ELISA) and in addition adaptations of them permit the use of microtitre plates meaning that small amounts of

reagents are required, therefore the cost of performing these assays can be cut. However although advances have been made in serological tests for toxoplasmosis no individual test is used extensively. Most tests have some drawbacks and further developments are required.

IMMUNOLOGY

Resistance to Toxoplasma infection is dependent on several factors; age, species and the host's immune response. Following infection with Toxoplasma gondii it appears that animals are protected from further infection (Frenkel, 1973). Considerable work has been carried out to elucidate the host immune response to Toxoplasma infection mainly in Man and murine species and while the picture is still incomplete it is clear that both humoral and cellular mechanisms are involved.

HUMORAL IMMUNITY

Antibody

Antibody to Toxoplasma can be detected in the serum of animals following infection with the parasite (Sabin and Feldman, 1948; Hartley, 1961; Huldt, 1966) and in some species this antibody is long lasting. In mice following experimental infection with T. gondii IgM was first detected on day 2 and IgG on day 8 of which IgG₂ was the major subclass (Handman and Remington, 1980). Passive serum transfer experiments have given varying results, possibly due to either different inoculation regimes or species differences. Hyperimmune guinea pig serum inoculated into mice did not protect against a lethal challenge (Foster and McCulloch, 1968). However immune rabbit serum inoculated either i.v. or i.p. 48 hours prior

to infection conferred significant resistance against lethal challenge as assessed by counts of tissue cysts in brains and the survival rate (Masihi and Werner, 1978). Transfer of human dye test negative serum to the same mouse strain gave partial protection suggesting that a mechanism other than antibody may be involved. Passive transfer of monoclonal antibodies raised against both surface and cytoplasmic antigens also protected against challenge infection (Johnson, McDonald and Neoh, 1983; Sharma, Araujo and Remington, 1984).

Treatment of mice with anti-IgM from birth abolishes antibody responses but does not effect cell mediated immunity. Mice treated in this way were infected with T. gondii and all survived the acute phase of the infection but succumbed by a mean of 40 days. Transfer of immune serum reduced the losses during the chronic phase by 50 per cent. This suggests that clearance of the organism by antibody during the early stages of the infection prevented death during the chronic phase (Frenkel and Taylor, 1982).

Several workers have tried to establish the mechanism by which antibody operates. Toxoplasma are able to survive in macrophage phagosomes by preventing fusion with the lysosome, however if they are incubated with specific antibody before being added to a macrophage population fusion of these vesicles occurs resulting in parasite death (Jones, Len and Hirsch, 1975). Moreover specific antibody can induce resident or elicited mouse peritoneal macrophages to phagocytose more rapidly and kill the intracellular organisms more efficiently (Shirahata, Shimizu and Suzuki, 1976; Jones et al, 1975). Similar results have been found with human

peripheral blood monocytes (Borges and Johnson, 1975). These effects are only seen if antibody is added to the system before the parasites have become intracellular (Jones et al, 1975). It appears that specific antibody opsonises the parasite during its extracellular phase enabling phagocytosis to occur more rapidly and preventing the parasite from entering by active penetration. Monoclonal antibodies raised against single membrane associated antigens of T.gondii are also capable of opsonising the organisms resulting in increased phagocytosis (Hauser and Remington, 1981).

Complement

In the presence of human specific antibody and fresh sera Toxoplasma organisms can be lysed (Sabin and Feldman, 1948) and this forms the basis of the dye test which is widely used in the diagnosis of toxoplasmosis. The component from fresh human sera required for this reaction is called "accessory factor", it is heat ~~labile~~ and is present in several species (Sabin and Feldman, 1948; Hartley, 1961). Although earlier studies in the late 1950's suggested that this accessory factor consisted of properdin and complement (Feldman, 1956), more recently a patient deficient in the C2 and C4 components of complement, that is two of the classical pathway substances, was found to have very low dye test activity. On the addition of C2 and C4 the activity rose considerably (Suzuki, Tsunematsu and Torisu, 1971). Other workers (Schreiber and Feldman 1980) demonstrated that sera which had been specifically depleted of the classical pathway components were unable to activate complement after a Toxoplasma infection. Since this process was dependent on Ca^{+} ions this provided further evidence of the involvement of the classical pathway (Suzuki and Kobayashi, 1985a).

From the available evidence accessory factor is likely to consist of the early initial components of the classical complement pathway. An in vivo role for accessory factor has also been shown, since the passive transfer of it together with immune serum to guinea pigs increased the survival time against a lethal challenge with T.gondii whilst antibody alone did not (Foster and McCulloch, 1968).

Thus the available evidence points towards humoral mechanisms having some influence on Toxoplasma infection but its effects are limited to the extracellular organism.

CELL MEDIATED IMMUNITY

T cells

Over the past twenty years it has become clear that cell interactions are an essential feature of the immune system and that an array of responses occur when antigen is introduced into the system. These interactions take place either by cell to cell contact or by secretion of soluble products from the cells.

Before T cells can be specifically activated they must recognise antigen in conjunction with class I molecules of the major histocompatibility complex (MHC) on the surface of antigen presenting cells. After this interleukin 1 (IL-1) produced by macrophages stimulates T cells to express receptors for the lymphokine interleukin 2 (IL-2) and also to produce this substance. Several populations of T cells exist including helper and suppressor cells and they can be activated under certain conditions although the criteria for this is not established. T helper cells also secrete two other lymphokines namely B cell growth factor

(BCGF) and B cell differentiation factor which induce B cells to differentiate into plasma cells.

One method of assessing the role played by T cells in the immune response to Toxoplasma infection is to use mice which are deficient in these cells. Nude athymic mice meet this criterion. Thus Lindberg and Frenkel (1977) took both nude athymic mice and their hirsute littermates, infected them with T.gondii and treated then with sulphadiazine drugs for three weeks. All hirsute mice survived after the treatment was stopped, whereas all the nude mice died (Mean survival time 6.3 days). Transfer of thymocytes during the drug treatment, had the effect of increasing survival time whilst bone marrow derived cells had no effect. Serum antibody levels were also found to be low suggesting that the humoral response is T cell dependent. Immune serum given after drug treatment had no effect, this suggested to the authors that antibody had no role in protective immunity, however no attempt was made to give sera earlier in the infection when its presence may have been more effective. In another study proliferative organisms were detected in acites fluid from Toxoplasma infected nude mice but not in infected hirsute littermates and the inflammatory reaction seen in the brains of the latter was absent in the nude mice giving further evidence for the importance of T cells in the control of infection (Buxton, 1980).

In vitro studies have shown that T cells from both human and murine species undergo blastogenesis in response to Toxoplasma antigens (McLeod, Van Le and Remington, 1982; Hughes, Gonzalez, Guhi and Hudson, 1985). Furthermore monoclonal antibodies to T cell subsets have demonstrated that it is the T helper/ inducer

cells which proliferate. However analysis of peripheral blood from patients with recent symptomatic toxoplasmosis revealed increased numbers of suppressor T cells, whereas asymptomatic toxoplasmosis had normal numbers (Luft, Kansas, Engleman and Remington, 1984). These findings raise the possibility that increased numbers of suppressor T cells correlate with the disease state.

Although T cell cytotoxic activity had been shown against cells coated with Toxoplasma antigen (Rose, Zeschke and Podleski, 1974), it seems unlikely that this mechanism played a role in vivo since a high cell to target ratio was required and no parasite antigen has been demonstrated on the surface of infected cells (Handman, Chester and Remington, 1980). Delayed type hypersensitivity (DTH) has been reported following Toxoplasma infection in hamsters (Frenkel, 1967) and in mice (Handman et al, 1980).

Studies in the last decade have demonstrated that a subset of T cells can transfer the DTH response. Furthermore these cells release lymphokines which activate macrophages, leading to an increased killing capacity of numerous intracellular pathogens. Considerable research has been carried out to establish the role of this response in Toxoplasma infected humans and animals.

Macrophages

Macrophages can readily be obtained from the peritoneal cavity of mice either by saline washings or by eliciting with various substances such as thioglycolate broth or glycogen saline or activation with an infectious agent. Human macrophages can be obtained by culture of perip^heral blood monocytes. By the use of

the proliferative tachyzoite stage of Toxoplasma to infect the cells, the ability of macrophages to phagocytose and kill the organism under various conditions can be observed by light microscopy. Activated peritoneal macrophages from hyperimmune mice have been shown to inhibit multiplication of tachyzoites and this activity was present even months after initial infection (Remington, Krahenbuhl and Mendenhall, 1972). Elicited macrophages also had this ability, although it lasted for only two to three months (Shirahata, et al, 1976).

Before human macrophages developed an enhanced killing capacity they required in vitro stimulation by immune lymphocytes which had been cultured with Toxoplasma antigen (Anderson and Remington, 1974), Jones and his coworkers (1975) reported the same requirements for resident mouse peritoneal macrophages. The supernatant from the lymphocyte culture was also capable of stimulating the macrophages and the active part of this supernatant was called Toxoplasma Growth Inhibition Factor (Toxo-GIF) (Shirahata et al, 1976; Shirahata, Shimizu, Noda and Suzuki, 1977). Production of this factor could be enhanced by culturing splenic lymphocytes with Concanav^alin A or PHA, (both T cell mitogens), whereas lipopolysaccharide (B cell mitogen) had no effect (Shirahata et al, 1977). It was also shown that antithymocyte serum could dimiⁿⁱsh Toxo-GIF production, as could protein synthesis inhibitors (Shirahata et al, 1977). Further characterisation revealed that Toxo-GIF was a glycoprotein with an isoelectric point between 4.9-5.9, it was unaffected by incubation at 56°C for 30 minutes and unstable at pH2. The molecular weight as determined by sephadex G100 was between 35-55 kD (Shirahata and Shimizu, 1979).

Similarities exist between the biochemical characteristics of Toxo-GIF and gamma interferon which is also produced by activated T cells.

Following infection of mice with a virulent strain of T.gondii a viral inhibitor could be detected in the serum. This activity reached a peak at 24 hours post infection and remained for at least 72 hours (Rytel and Jones, 1966). Culture of lymphocytes with Toxoplasma antigen gave a substance which had both Toxo-GIF and anti-viral activity (Shirahata and Shimizu, 1980; Sakurai, Takei, Omata and Suzuki, 1981). Type 1 interferon which was induced in L cells by infection with Newcastle Disease virus did not affect the multiplication of the parasite (Shirahata and Shimizu, 1980; Pfefferkorn and Guyre, 1984). Furthermore the Toxo-GIF activity could not be removed by anti-interferon type 1 antibody (Sakurai et al, 1981). In vivo gamma interferon activity could be detected four-seven days post infection with a peak at day 14, the difference in the timing compared to Rytel's study may be due to the generation time of avirulent strains being longer than virulent ones (Shirahata and Shimizu, 1980). Recombinant gamma interferon inhibited Toxoplasma growth in human fibroblasts, but had no effect on extracellular organisms (Pfefferkorn and Guyre, 1984). In vivo murine recombinant gamma interferon protected mice from a lethal challenge, with both higher antibody titres and enhanced macrophage activity (McCabe, Luft and Remington, 1984).

It is not yet certain whether Toxo-GIF and gamma interferon are identical, however from the available information it appears that they are. What is clear is that lymphokines released by T cells are able to increase the in vitro killing capacity of

macrophages. Furthermore studies with recombinant gamma interferon demonstrate that it is also effective in vivo.

Macrophage migration inhibitory factor (MIF) has also been obtained from immune splenic lymphocytes and in vivo is present at greatest concentrations three to four weeks after infection (Igarashi, Taguchi and Suzuki, 1979). The separation of supernatant from immune spleen cell culture on a sephadex G100 column gave two peaks with molecular weights of 30-40 kD and 3-5 kD (Igarashi et al, 1979). Toxo-GIF activity was found in the higher molecular weight peak, while only MIF activity was detected in the other peak (Nagasawa, Igarashi, Matsumoto, Sakura, Marbella and Suzuki, 1980).

Whether MIF is a separate entity or merely another manifestation of gamma interferon activity remains to be seen. If this was the case, then it may be that the low molecular weight compound is a breakdown product which has lost its Toxo-GIF activity but not its MIF properties.

Although not completely understood the mechanisms by which macrophages can kill Toxoplasma are beginning to be elucidated. Tachyzoites are resistant to killing by either H_2O_2 and O_2 possibly due to them having endogenous catalase (Murray and Cohn, 1979). However evidence exists that H_2O_2 and O_2 are precursors for oxygen intermediates OH and O_2 which seem to be responsible for the killing capacity of the macrophages (Murray and Cohn, 1980). Studies with monocyte derived macrophages from chronic granulomatous disease patients (CGD) and mouse L cells, demonstrated that killing of T.gondii could still occur although no

respiratory burst could take place (Wilson, Tsai and Remington, 1980; Murray, Byrne and Rothermel and Cartelli, 1983). This suggested that oxygen independent mechanisms could also be responsible for the death of the organism. Gamma interferon has been shown to increase macrophage killing of T.gondii by both these mechanisms (Murray and Cohn 1980; Murray et al, 1983).

Since Toxoplasma is able to infect both phagocytic and non-phagocytic cells it is important that gamma interferon can also inhibit parasite growth in fibroblast cells. A possible mechanism for this action is by the induction of non-phagocytic cells to degrade tryptophan. Whether the metabolites of this degradation are toxic for Toxoplasma or whether the organism is starved of an essential amino acid is not yet established (Pfefferkorn, 1984). Recent studies with tissue cysts cultured in vitro have shown that although gamma interferon does not promote cyst formation and is not a requirement for cyst development it provides a means of preventing cell destruction by the expanding population of tachyzoites and it allows the persistence of cysts without rupture for prolonged periods (Jones et al, 1986).

Natural Killer Cells

Another action of gamma interferon is to enhance natural killer cell activity and it seems possible that they may play a role in host defences against Toxoplasma infection. Acute toxoplasmosis in mice has been shown to induce splenic NK cells that are cytotoxic for tachyzoites in vitro (Hauser and Tsai, 1986). Subcellular components of T.gondii have been shown to increase human NK cell activity (Sharma, Verhoef and Remington, 1984).

IMMUNOSUPPRESSION

In order to achieve a maximum survival rate parasites must be constantly adapting to their environmental conditions. One facet of this adaptation is evasion of host immunity and T.gondii achieves this in part by preventing fusion of the vesicles within the macrophage (Jones et al, 1972). Several authors have proposed that it is also able to achieve immune evasion by immunosuppressing its host.

Various workers have reported that infection with T.gondii can induce suppression of primary antibody responses to both Toxoplasma and unrelated antigens (Huldt, Gard and Olovson 1973; Strickland, Pettit and Voller, 1973; Suzuki, Watanabe and Kobayashi, 1981a). Suppression of the response to live viral infection can also occur (Buxton, Reid, Finlayson and Pow, 1980). This suppression is not Ig class specific and responses to both T cell dependent and independent antigens can be affected (Suzuki et al, 1981a). Secondary antibody responses to unrelated antigens can also be altered by infection with T.gondii (Suzuki, Watanabe and Kobayashi, 1981b). However studies have shown that it is the initiation of memory cells which is affected and not the expression^S of memory, since infection with the parasite after the initial priming has taken place has no effect on the antibody response (Suzuki and Kobayashi, 1985b). The effector mechanism of this suppression appears to be dependent on a plastic adherent, radiation resistant cell (Suzuki and Kobayashi, 1981). Further studies were able to define the cell as a macrophage since its effect could be abrogated by silica or carageenan which are selectively cytotoxic for macrophages (Suzuki and Kobayashi, 1985b).

Genetic control involving both MHC linked and non MHC linked genes has been shown to be important in this suppression in studies with various inbred mouse strains (Suzuki and Kobayashi, 1985). This may explain why some workers have been unable to find Toxoplasma induced suppression.

VACCINATION

At the present time there is no effective immunoprophylaxis for toxoplasmosis in either man or animals. However several workers have attempted to immunise experimental animals against T.gondii infection. Live vaccines are effective, however since the parasite remains within the host and may be reactivated under certain conditions the use of the type of vaccine, in man at least is unacceptable.

Studies with killed preparations have had varying degrees of success. Immunisation of rabbits with heat killed organisms followed by challenge with the virulent RH strain was unsuccessful (Huldt, 1966), whilst Foster and McCulloch (1968) found that guinea pigs could be protected from a virulent challenge by immunisation with phenol killed parasites in Freund's complete adjuvant (FCA). However the statistical significance of the result was not tested and adjuvant appeared to play an important role in the success rate.

Other workers used a different virulent challenge strain and had more success. Formalin inactivated tachyzoites, soluble and particulate fragments of the organism and its RNA were all able to give some protection against challenge infection but it was incomplete and short lived (Krahenbuhl, Ruskin and Remington, 1972).

More recently temperature sensitive mutants of T.gondii have been isolated (Pfefferkorn and Pfefferkorn, 1976). One of these ts-4, is non-lethal to mice even when given in high numbers. It could not be isolated from infected mice two months after the initial inoculation indicating that it is a non-persistent organism. Mice infected with this strain could resist a substantial challenge three months after infection and a more limited challenge one year later (Waldeland and Frenkel, 1983).

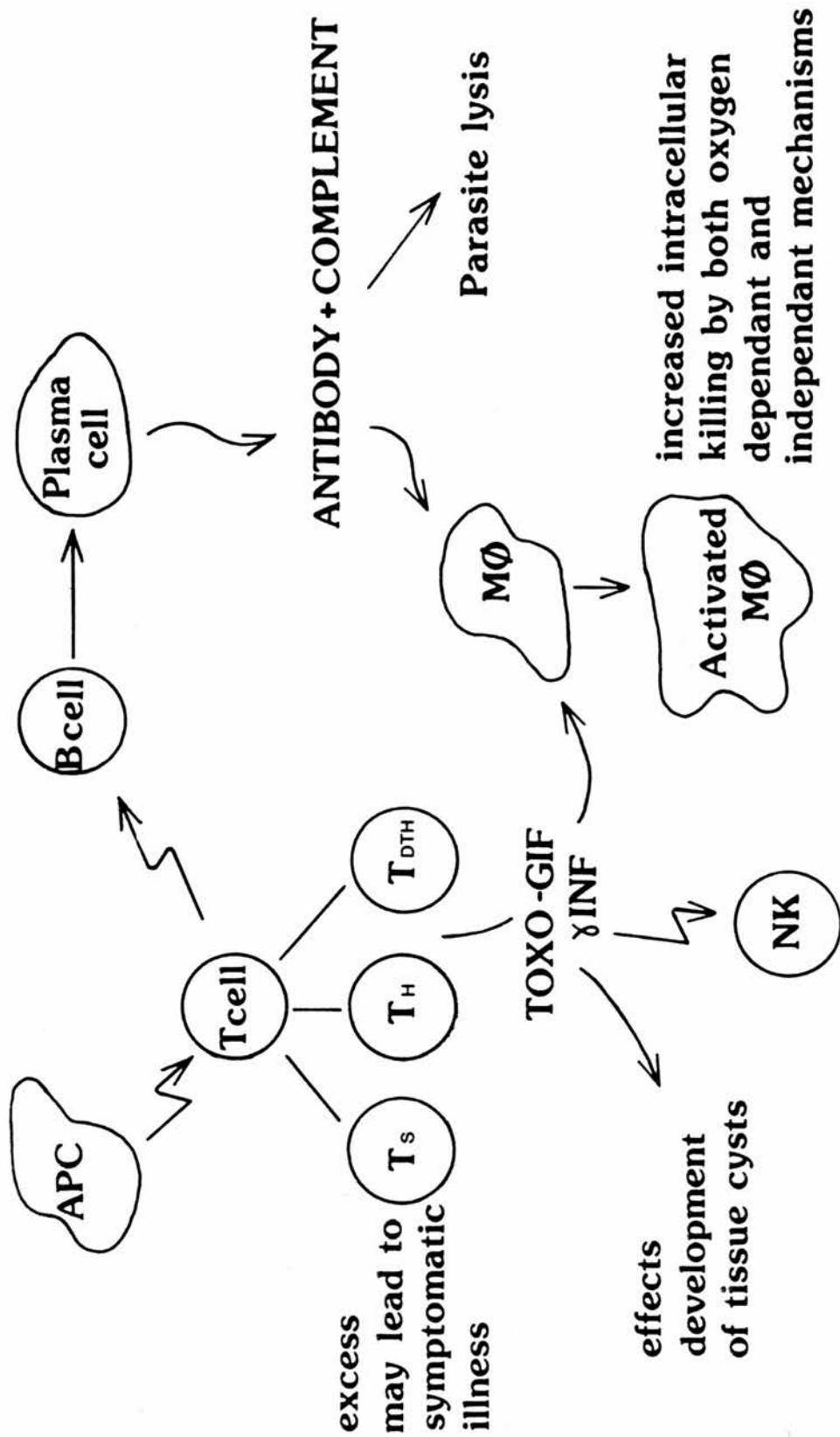
Immunisation with γ -irradiated tachyzoites gave some short lived protection against a virulent challenge which was more substantial to that seen with killed organisms (Seah and Hucal, 1975; Chhabra, Mahajan and Ganguly, 1979).

The differences in data for killed preparations of T.gondii can be explained by species differences, use of adjuvant and variations in the challenge infection. Alteration of parasites by irradiation or the use of non-persistent strains of T.gondii are perhaps the most encouraging reports in the field of vaccine development at the present time, however the development of a efficient immunisation programme to control toxoplasmosis is still far away.

A summary of the host's immune response to T.gondii infection as understood at the present time is shown in figure 1.3

FIGURE 1.3 Synopsis of host's immune response to Toxoplasma infection

T cells activated by Toxoplasma antigens may have several effects, for example providing help for antibody production. Specific antisera may act with complement to cause parasite lysis or promote phagocytosis of opsonised organisms. Lymphokines released by T_H or T_H/DTH cells can activate other effector cells in the immune system e.g. macrophages and NK cells to promote parasite killing. In addition imbalance in the ratio of T_S to T_H may lead to symptomatic disease.



ANTIGENIC STRUCTURE

It is important to characterise and isolate the antigens of Toxoplasma for several reasons:

1. To establish which antigens elicit an immune response in the host.
2. To permit an accurate comparison between existing serological tests used in the diagnosis of toxoplasmosis and in the development of new systems.
3. To aid the development of either conventional or subunit vaccines.

Various techniques and antigen preparations have been used to try and elucidate the antigenic structure of Toxoplasma. The majority of these have concentrated on the tachyzoite stage of the life cycle since it can be obtained in large numbers from serial passage in mice or rats.

Immunodiffusion methods for the analysis of T.gondii antigens have given between one and three bands (Chordi, Walls and Kagan, 1964; Strannegard, 1962). Immuno-electrophoresis studies permitted further separation of these bands, and when either a sonicated lysate or a freeze-thawed preparation was used three or four bands could be distinguished (Chordi et al, 1964; Hughes and Balfour, 1981). Another study which used the same technique to examine both the pellet and supernatant of a freeze thawed antigen preparation detected 10 antigens (Yano and Nakabayashi, 1980). However not all of these bands could be detected by any one of several rabbit antisera used. Treatment of the parasite with two non-ionic

detergents simultaneously, namely saponin and octyl glucoside, resulted in 11 antigens being detected by immunoelectrophoresis. When the isoelectric point of each antigen was calculated by isoelectric focusing, it was found that eight had acidic isoelectric points while the remaining three antigens did not resolve, possibly due to pH sensitivity. Molecular weights were estimated using Sephacryl S300 and were between 10-324kD. These were higher values than expected and were possibly due to the presence of the detergent (Hughes and Balfour, 1981).

Other workers separated tachyzoites into a soluble and an insoluble fraction and analysed them by PAGE (Johnson, McDonald and Neoh, 1981). Nine polypeptides were found in the soluble fraction (molecular weights 20-133 kD). A further eight were present in the insoluble fraction with molecular weights from 28-80 kD. Figure 1.4 is a diagrammatic representation of Toxoplasma polypeptides as described by several workers.

Labelling of parasites with radio isotopes can be a useful way of studying an organism's antigenic composition. Handman, Goding and Remington (1980) labelled live cultured tachyzoites with [³⁵S]. Following this the tachyzoites were lysed by freezing and thawing and the antigens were then precipitated with antisera from mice chronically infected with T.gondii. This gave a complex picture, with one dimensional gel electrophoresis detecting 32 bands. In the absence of the antisera this antigen preparation gave more than 1000 spots on 2 dimensional gel electrophoresis.

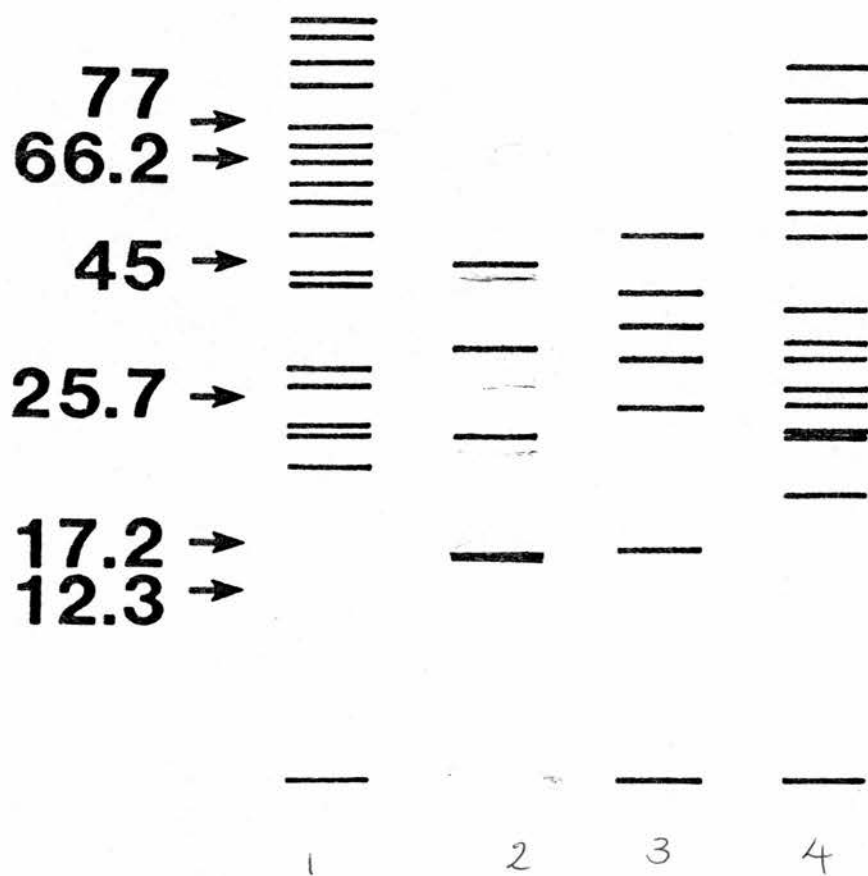
FIGURE 1.4 Diagram^matic representation of the molecular weights of Toxoplasma antigens obtained by several techniques.

1. Whole tachyzoites separated by PAGE (Johnson et al, 1981).

2. Membrane proteins labelled by ¹²⁵I (Handman et al, 1980).

3. Toxoplasma antigens recognised by human IgM by western blotting (Sharma et al, 1983).

4. Toxoplasma antigens recognised by IgG antibodies (Sharma et al, 1983).



Fortunately surface labelling of tachyzoites with the lactoperoxidase technique gave a much simpler picture. Four bands could consistently be detected with molecular weights of 43,35,27 and 14 kD (Handman et al, 1980). Further studies with monoclonal antibodies showed that the 27 and the 43 kD antigens could be precipitated by monoclonal antibodies 1E11 and 1E3 respectively (Handman and Remington, 1980). Two monoclonal antibodies were able to precipitate both the 35 and the 14 kD antigens. Since treatment with mercaptoethanol did not alter this result it is unlikely that they are linked by disulphide bonds. It is possible that the 35 kD is a dimer of the 14 kD antigen or that they are covalently linked.

Other workers used both the lactoperoxidase and the Iodogen methods to label the tachyzoites and while there were significant differences between the two methods, both gave a band with molecular weight 30 kD. A monoclonal antibody raised against this antigen P30 was used in its purification by immunoabsorption. This allowed it to be characterised as a protein with an extensive hydrophobic region suggesting that it is likely to be a membrane protein (Kasper, Crabb and Pfefferkorn, 1983).

Separation of denatured antigens by gel electrophoresis is a valuable technique for the analysis of complex protein mixtures because of its capacity for high resolution. Antigens separated in this manner can then be transferred to nitrocellulose paper by electro-blotting (Towbin, Staehelin and Gordon, 1979), and with specific antisera immunologically reactive components can be detected. This technique has been used by several workers to identify antigens detected by human sera. Sera obtained from acutely infected patients *were* used to probe a sonicated lysate of

tachyzoites. Two antigens, one 35 kD the other 50 kD were detected by IgM (Partaen, Turunen, Passivuo, Forsblom, Suni and Leinikki, 1983). Further work from this laboratory which analysed sequential serum samples from a patient with acute toxoplasmosis permitted the detection of eight antigens by IgM, with molecular weights that ranged from 6 to 50 kD (Partaen, Turunen, Passivuo and Leinikki, 1984). These responses disappeared over a period of weeks and this may explain why only two antigens were detected in the previous study. When an anti-IgG conjugate was used with this sera, numerous bands were detected (Partaen et al, 1984). With a different antigen preparation other workers found that IgM detected antigens with molecular weights of 6, 22, 32, 45, 66 kD, however not all these antigens were detected by each serum (Sharma, Mullenaz, Araujo and Erilichand and Remington, 1983). Differential centrifugation gave enrichment of the 6, 22, and 32 kD antigens, which suggests the possibility that they are membrane proteins (Sharma et al, 1983).

Initial studies have been carried out to determine the nature of the surface components of T.gondii. Speculation that polysaccharides and glycoproteins were not present on the surface of the parasite arose when binding of fluorescein-conjugated lectins to intact tachyzoites could not be detected, since lectins are normally considered to bind to both polysaccharides and glycoproteins (Sethi, Rahman, Pelster and Brandis, 1977; Handman et al, 1980). Subsequent lectin studies have shown that glycosylated polypeptides are present but in low concentrations and can only be detected in the insoluble fraction obtained after ultracentrifugation (Johnson, Haynes, Leppard, McDonald and Neoh,

1983; Sharma et al, 1983). Failure to detect them in the former studies may have been due to the insensitivity of the assay system used. Further evidence for the presence of surface carbohydrate bearing molecules came from positive periodic acid-Schiff staining of antigens separated by either immunoelectrophoresis (Hughes and Balfour, 1981) or western blotting (Sharma et al, 1983). It was found that oxidation of the antigen preparation with sodium periodate affected the antigen profile detected by human IgM and IgG antibodies (Sharma et al, 1983; Naot, Guptill, Mullenaz and Remington, 1983) and that a 6 kD antigen was removed by this treatment (Sharma et al, 1983).

Coomassie blue staining of antigens separated by immunoelectrophoresis gave six positive bands, indicating that proteins are present (Hughes and Balfour, 1981). Treatment of the antigen preparation with pronase or trypsin removed both a 32 and a 22 kD antigen (Sharma et al, 1983). Prote⁰lysis and heat alters the antigens that can be detected by both IgM and IgG human antibodies (Naot et al, 1983; Sharma et al, 1983).

A lipid component was detected by Black Sudan staining (Hughes and Balfour, 1981). However treatment of the antigen preparation with lipase did not effect the amount of binding by either IgM or IgG (Sharma et al, 1983; Naot et al, 1983). Furthermore when a lipid isolate from a Toxoplasma sonicate was used in an ELISA system no IgM or IgG response could be detected (Naot et al, 1983). It may be that a lipid component is present but in very small amounts or that the particular sera used in this detection system can not recognise it.

Very little is known about the antigenic differences between T.gondii tachyzoites, bradyzoites and sporozoites. However one study which used fluorescence techniques showed that antisera raised to tachyzoites could react with both tachyzoites and bradyzoites whilst the reverse was not true (Lunde and Jacobs, 1983). Monoclonal antibodies were raised against stage specific antigens of T.gondii sporozoites and analysis by gel electrophoresis revealed that sporozoites had two major membrane antigens with molecular weights 67 kD and 25 kD (Kasper, Bradley and Pfefferkorn, 1984). Human sera from an outbreak of toxoplasmosis, due to the ingestion of oocysts, was shown to contain antibodies specific for these sporozoite antigens (Kasper and Ware, 1985), whereas sera from mice infected with the tachyzoite stage were negative (Kasper et al, 1985).

T.gondii has a complex antigenic structure and although variations exist in data from different laboratories it appears that there are at least four surface antigens. Both protein and carbohydrate moieties are present in the membrane, however it has not been established which form they are present in. At this time it is unclear whether a lipid component is present.

Western blot studies with human sera demonstrate that the IgM response recognises fewer antigens and disappears more rapidly than does the IgG response.

Differences between the stages in the parasite's life cycle are beginning to be elucidated, however much more work is required.

OVINE TOXOPLASMOSIS

HISTORY

While toxoplasmosis in sheep is most commonly manifest as a cause of abortion the first two cases of toxoplasmosis described involved the central nervous system (CNS). In both there was a non-suppurative encephalomyelitis and organisms with the morphology of Toxoplasma were present (Olafson and Monlux, 1942; Wickham and Carne, 1950). It was not until the 50's that it was realised that T.gondii could cause abortion in sheep.

In 1954 an outbreak of ovine abortion occurred in New Zealand. Typically 4-6 weeks before lambing sporadic abortions took place followed by an abortion storm at the onset of parturition. Losses on the properties involved ranged from 3-50 per cent (Hartley et al, 1954). While the foetal membranes were clear the cotyledons were covered in white spots equivalent to foci of necrosis. Small numbers of intra and extra-cellular bodies 1-4 μ m in length were also occasionally seen. Attempted transmission of the infection to pregnant ewes resulted in abortion in 25 per cent of the animals, while mice infected with the same material showed no signs of disease. Following successful experimental transmission to mice in subsequent seasons this New Zealand Type II abortion as it was initially called, was confirmed as being due to Toxoplasma gondii (Hartley and Marshall, 1957).

Outbreaks of Toxoplasma abortions have since been reported in many countries among them Britain (Beverley and Watson, 1959); Australia (Osborne, 1959); Canada, (Hulland and Tobe, 1961); Ireland (Crowley, 1964); Norway (Waldeland, 1976b) and Colombia

(Perry, Mongollon, Grieve and de Galvis, 1979). Little information exists on the actual losses due to Toxoplasma abortions however Hartley in New Zealand estimated that it was responsible for 74 per cent of abortions, whilst in both Norway and Britain it was thought to be the cause of 20 per cent of ovine foetal death (Waldeland 1976b; Beverley and Watson, 1962; Blewett and Watson, 1984). As stated previously serological surveys of sheep have demonstrated that sheep have a high rate of Toxoplasma infection.

It is thus apparent that there is a discrepancy between the prevalence of antibody to Toxoplasma and the incidence of diagnosed cases of clinical toxoplasmosis. It seems likely that certain criteria are necessary before congenital transmission takes place and that most infections outwith pregnancy are asymptomatic.

Both naturally occurring and experimentally induced infections have helped elucidate the factors involved in Toxoplasma abortion. Infection in non-pregnant sheep normally goes unnoticed (Hartley, 1961) however an initial febrile response does occur (Smith, 1961; Blewett et al, 1982; Buxton and Finlayson, 1986) and this almost certainly coincides with a transient parasitemia (Jacobs and Hartley, 1964; Reid, Buxton, Gardiner, Pow, Finlayson and Maclean, 1982).

If primary infection occurs during pregnancy several outcomes are possible depending on the age of the foetus at the time of infection. In one experiment sheep were infected before 30 days gestation with either T.gondii tachyzoites or tissue cysts (Jacobs and Hartley, 1964). A considerable number of these appeared non-pregnant and on examining their uteri, signs of resorption were



seen in at least ten of the seventeen ewes. It has been demonstrated by several workers that infection during mid pregnancy leads to foetal death in utero with the subsequent mummification of the foetus. In addition the mortality rate is greater after infection at mid gestation than at a later stage of pregnancy. Following infection between 76-88 days gestation only two out of 14 live lambs were produced compared with 11 out of 15 at 115 days gestation (Miller, Blewett and Buxton, 1982). Although ewes infected with Toxoplasma after 120 days of gestation gave birth to live lambs all the foetal membranes were infected with the organism (Watson and Beverley, 1971).

No evidence was found that lateral transmission could occur between pregnant animals running on the same pasture as experimentally infected ewes (Hartley 1961; Jacobs and Hartley, 1964; Teale et al, 1982). This was also found to be true in field cases (Waldeland, 1976b).

In several studies it was shown that infection of the ewe prior to mating would not lead to infection of the foetus (Hartley, 1961, Jacob and Hartley, 1964; Buxton and Finlayson, 1986). Furthermore in another experiment in which sheep were infected two years before challenge with T.gondii in mid pregnancy no signs of foetal infection could be demonstrated (Beverley and Watson, 1971). In addition when ewes were inoculated seven weeks prior to mating and then challenged at 90 days gestation only one out of 14 ewes gave birth to a lamb infected with Toxoplasma. The finding that one ewe did abort may be due to the i.v. route being used for infection (Beverley and Watson, 1971).

Following initial infection with T.gondii sheep can remain infected for long periods of time. In one experiment Hartley and Moyle (1974) studied a group of sheep for seven years. They found that seroconversion to T.gondii infection occurred in 51 per cent of the animals and latent infection was detected in 93 per cent of this group at the end of the seven year period. No congenital toxoplasmosis was detected in the offspring of any of these sheep (Hartley and Moyle, 1974). Watson and Beverley (1971) demonstrated that 88 per cent of ewes which had aborted due to toxoplasmosis in the previous season gave birth to normal lambs when challenged at mid pregnancy. Again the i.v. route was used and this may account for the figure being less than 100 per cent. Precolostrum serum samples from lambs of chronically infected ewes were all negative for anti-toxoplasma antibody (Munday, 1972) indicating that the foetuses were not infected.

From the above findings it appears that infection with T.gondii must occur during pregnancy for congenital transmission to take place. Furthermore following infection sheep are protected from further challenge and will not abort in subsequent pregnancies.

TRANSMISSION

After T.gondii was found to be a causative agent in ovine abortion several modes of transmission were postulated although none of them completely explained either the experimental or field findings. One idea was that transmission occurred by ingestion of tissue cysts coughed up from the lungs of chronically infected sheep or other animals (Jacobs and Hartley, 1964), however tissue cysts have not been demonstrated in the saliva of sheep. Ingestion

of infected foetal membranes can cause congenital infection (Hartley and Marshall, 1957). Due to synchronised breeding in most flocks all ewes lamb together over a relatively short time span and although infection at this time may lead to vertical transmission, it would not account for the abortion storms commonly seen. Another possibility was venereal transmission and infectious Toxoplasma has occasionally been isolated for semen (Spence et al, 1969, Teale et al, 1982). However mating of seronegative ewes with a seropositive male did not lead to an antibody response or infection in the ewes (Blewett, et al 1982). Even if venereal transmission did occur Toxoplasma would gain entry to the foetus very soon after conception and would probably cause foetal resorption and infertility rather than abortions. This does not reflect either field or experimental observations.

It was not until the discovery that T.gondii was a coccidian parasite with the cat family as its definite host that some light was shed on the possible means of spread to herbivores (Hutchison et al, 1970).

Oocysts excreted in cat faeces have been shown to be just as effective at producing abortions as tissue cysts (Beverley, Hutchison, Allsup, Spence and Watson, 1975). Several outbreaks of toxoplasmosis have been reported where good circumstantial evidence exists that oocysts were the source of infection. Following an outbreak of Toxoplasma abortion the cats on the farm were checked. Nine out of sixteen cats tested had a chronic Toxoplasma infection whilst one was actively shedding oocysts (Dubey, Miller, Powell and Anderson, 1986). A similar finding had previously been reported by Plant, Ricardson and Moyle, (1974). However difficulties arise

when trying to show that the oocysts present in cat faeces are actually responsible for the outbreak in sheep. The time from initial infection in the cat to the demonstration of ovine abortion storms due to toxoplasmosis can be over 18 months. Therefore when cats are tested after an outbreak even if they are found to be actively shedding oocysts it is extremely unlikely that they are the source of infection. Thus it would be more relevant if efforts were made to isolate oocysts from the bedding, pasture, feed and water surrounding the sheep.

IMMUNOLOGY

The early reports on T.gondii infection involving the CNS gave the first indications that the parasite could evoke an ovine immune response (Olafson and Monlux, 1942; Wickman and Carne, 1950). Although unrecognised at the time the marked cellular infiltrate in the areas of the lesion consisted of mononuclear phagocytes and lymphocytes. After Toxoplasma had been identified as a causative agent of ovine abortion, serological tests were performed to determine if specific anti-toxoplasma antibody was present.

It was found that in both experimental and natural cases of toxoplasmosis seroconversion occurred (Hartley and Marshall, 1957; Beverley and Watson, 1959). Ewes that had either aborted or given birth to congenitally infected lambs had higher antibody levels than ewes in the same flock, which lambed normally. When ewes with high antibody titres were challenged with T.gondii during pregnancy subsequent examination of the placenta and the foetus did not reveal infection (Jacobs, 1961; Beverley and Watson, 1971; Blewett et al, 1982; Buxton and Finlayson, 1986). However experimental i.v. inoculation of a large dose of proliferative organisms did

lead to infection of the foetus, presumably by reaching and invading the uterine tissues before the ewe had time to mount an adequate immune response (Jacobs and Hartley, 1964). Specific antibody was first detected by IHA approximately 10 days after experimental infection with tissue cysts and this belonged to the IgM class. (Miller et al, 1982; Blewett, Bryson and Miller, 1983). Titres continued to rise and after a month the IgM antibody declined and was replaced by IgG. High levels of antibody persisted for long periods of time, often several years following both natural and experimental infection. At birth the presence of specific IgM or rising titres of IgG antibodies in the serum of lambs is indicative of congenital infection (Watson and Beverley, 1971).

To further dissect the ovine humoral response to the parasite, histopathological studies of lymph nodes from infected animals were carried out. In both foetal and adult lymph nodes numerous blast cells which contained either IgM or IgG were found (Buxton, Miller, Finlayson and Wallace, 1981; Buxton and Finlayson, 1986). In adult animals these blast cells were present from at least nine days post infection and specific anti-toxoplasma antibodies could be detected from day eight onwards (Buxton et al, 1981). In a study of the pathogenesis of toxoplasmosis in the ovine foetus IgM containing cells could be detected in the foetal lymph nodes 20 days after the ewe had been infected (Buxton and Finlayson, 1986). By day 30 the blast cells contained IgG and it was at this time that specific antibody to T.gondii could be detected in the foetal serum (Buxton and Finlayson, 1986).

Very little work has been carried out on the mechanisms of cellular immunity in ovine toxoplasmosis. However histological studies of lymph nodes after infection with T.gondii have suggested that a cell mediated response does play an important part in immunity to the parasite.

The earlier observations of an inflammatory reaction have now been corroborated and extended in both adult and foetal animals (Sharma and Gautam, 1978; Buxton and Finlayson, 1986). Lesions in brains of stillborn lambs consist of two types, those not directly caused by the organism and those which are and thereby evoke an immune response. Leucomalacia and white matter spongy changes have been demonstrated and it has been suggested that these are due to anoxia (Buxton, Gilmour, Angus, Blewett and Miller, 1982). Characteristic of foetal infection is the presence of mild perivascular cuffing by lymphoid cells and discrete foci of mononuclear cells with occasional Toxoplasma organisms present on their periphery (Buxton et al, 1982). As well as the lesions in the brain some foetal livers display accumulations of lymphoreticular cells (Buxton et al, 1982) and focal hepatic granulomata (Hartley and Karter, 1963). This cellular response is age dependent since 70 day old fetuses which had been infected at day 40, were unable to mount an inflammatory response and only foci of tissue necrosis were observed (Buxton and Finlayson, 1986).

As in the brain, the placenta of older fetuses had inflammation around the sites of cell destruction (Buxton and Finlayson, 1986). This necrosis is progressive and may eventually become calcified (Beverley, Watson and Payne, 1971).

From the available evidence it appears that following Toxoplasma infection a parasitaemia occurs, this provokes an immune response of which the antibody is the most easily measured part. For reasons which are not fully understood the organism remains in the brain and muscle of the animal for life and the animal is protected from further challenge. If however the ewe is pregnant when initially infected it seems possible that the following sequence of events occurs. Following a maternal parasitaemia tachyzoites reach the pregnant uterus. The organisms invade the gravid uterus where they parasitise and multiply in the cells of the caruncular septa. Focal necrosis occurs and invasion of the trophoblast follows (Buxton and Finlayson, 1986). The foetus can succumb to the original parasitaemia if infection occurs early in pregnancy and it may undergo resorption. If the foetus is old enough, then it may evoke an immune response which in some instances can be life saving, however death may still occur and in these cases it is thought that anoxia due to cotyledonary damage is the most probable cause (Buxton et al, 1982).

CHAPTER 2

Materials and Methods

ANIMALS

Mice

Mice used were either Swiss white or Porton outbred strains of both sexes from Toxoplasma free colonies and were at least four weeks old at the start of experimentation.

Sheep

Both male and female sheep of various ages and breeds were used in the study. More specific details are recorded in the relevant chapters.

Cats

Toxoplasma free cats of both sexes and under one year of age were obtained from either the Universities of Newcastle or Glasgow.

INOCULATIONS

In mice, intraperitoneal (i.p.) injections were given without anaesthesia with either a 16 gauge or a 21 gauge needle. The infection was into the abdomen, slightly to the right of the umbilicus.

In sheep, subcutaneous (s.c.) injections were given without anaesthesia with a 16 gauge needle. The injection site unless otherwise stated was behind the point of the shoulder. In order that an oral dose could be given efficiently, a gag was inserted into the ewes mouth and a stomach tube measuring 45 cm, passed down the oesophagus. The required dose in 5 ml HBSS was administered from an attached syringe and was flushed down with 20ml of distilled water from a separate syringe connected to the stomach tube by means of a 2- way tap.

TOXOPLASMA GONDII

Tachyzoites

The RH strain of T.gondii originally isolated from a fatal human encephalitis case (Sabin, 1941) was maintained in mice by serial passage every three days. A suspension containing 2×10^5 organisms was inoculated i.p. and on day 3 after infection mice were killed with carbon dioxide gas CO₂ and the exudate in the peritoneal cavity collected into a heparinised container. Cryopreservative which contained 20 per cent DMSO, 40 per cent normal rabbit serum and 40 per cent PBS was added to aliquots of the RH strain tachyzoites. They were left at room temperature for 30 minutes to allow the penetration of the preservative and then stored in liquid nitrogen.

Bradyzoites within Tissue cysts

The M1 strain of T.gondii originally isolated from an aborted ovine foetus was maintained by serial passage in mice every three to six months. Following decapitation their brains were removed aseptically and homogenised in (HBSS) by passage ten times through a 16 gauge needle. Between 40-100 tissue cysts were inoculated i.p.

Oocysts

To avoid contamination of the environment with infective oocysts the following procedure was used. A negative pressure isolator made of anodised aluminium with a perspex front (Fig. 2.1) was constructed (J.C. Forbes, Edinburgh, Scotland). The isolator which measured 173 x 73 x 71 cm was ventilated by a blow unit (Model BTC26, Airflow Development, High Wycombe, England) through

FIGURE 2.1 Negative pressure isolator with a cat
cage inside.



stainless steel filters (J.C. Forbes, Edinburgh, Scotland). Material could be placed in or removed from the isolator by means of a circular port at one end. This entry chamber measured 31 cm in diameter and 38 cm in length and had both an inner and outer cap. Before the objects were removed from this chamber they were sprayed with two per cent peracetic acid and left for 30 minutes to permit decontamination to take place.

Two pairs of heavy duty gloves and sleeves were incorporated into the front perspex panel, which allowed the operator to carry out manipulations within the isolator.

The cat was confined to an anodised aluminium cat cage which measured 105 x 52 x 52 cm (Northkent Plastics Ltd, Dartford, England) within the isolator. Sliding doors and a removable vertical partition to permit easy access to the cage and safe feeding and cleaning out facilities were added at MRI.

Cats were fed KiteKat and given fresh water to drink daily. They were allowed to adjust to their environment for at least two days before being fed a suspension of 1000 Ml strain tissue cysts mixed in a small amount of food. Their faeces were collected daily, placed in kilner jars before removal from the isolator and examined for presence of T.gondii oocysts. To achieve this a 5 ml sample of faeces was taken, mixed with 10 ml distilled water and centrifuged at 1000 x g for five minutes. Ten ml of saturated NaCl solution was added to the pellet and the mixture centrifuged at 1000 x g for three minutes. The top one ml of the supernatant was removed and examined microscopically for the presence of oocysts. If the sample contained oocysts the remainder of the faeces was

processed in a similar manner. To permit sporulation the oocysts were suspended in two per cent H_2SO_4 , and stored at $22^\circ C$ for two days, after which they could be kept at $4^\circ C$ for several months.

After the removal of the cat, the isolator and its contents were sterilised with two per cent peracetic acid for 12 hours, before being reused.

IRRADIATION OF TOXOPLASMA GONDII OOCYSTS

A 4000 Curie Caesium 137 source was used for the purpose of γ -irradiation. Doses were estimated with reference to previously exposed standard lithium fluoride discs and from exposure of an ionisation chamber of a Farmer dosimeter, with adjustments made to compensate for radioactivity loss due to the half-life of the isotope.

The oocysts suspension to be irradiated was placed in a plastic bijou to a depth of 0.7 cm which is equivalent to 1.2 ml. The midpoint of the suspensions were placed at a distance of 25.15 cm from the centre of gravity of the source. At this distance the irradiation times for 5 krad was 904-911 seconds. Higher doses were achieved by increasing the exposure times.

SERUM

Mice were killed with CO_2 and their major blood vessels severed. Blood was collected by pipette, placed in Eppendorf tubes, allowed to clot and retract and was then centrifuged. The serum was removed and stored at $-20^\circ C$

Blood from sheep was collected from the jugular vein into evacuated "vacutainer" tubes (Becton-Dickinson Ltd, Wembley,

Middlesex) and allowed to clot. Following retraction of the clot the serum was obtained after centrifugation at 2000 rpm for 15 minutes and stored at -20°C .

PREPARATION OF SPECIFIC ANTISERA

Antiserum against sheep IgM was raised in rabbits and the IgG fraction isolated by affinity chromatography on protein A Sepharose CL4B (Pharmacia Ltd., Milton Keynes, England). The IgG fraction was rendered monospecific by affinity chromatography on sheep IgG₁ and IgG₂ Sepharose 4B and foetal lamb serum Sepharose 4B. Antiserum against sheep IgG₁ and IgG₂ was raised in pigs and the IgG fraction prepared by ion exchange chromatography (Whatman DE52). Anti-light chain antibodies found to be present in this fraction were removed by affinity chromatography on sheep F(ab)₂ Sepharose 4B.

Affinity purified Pig IgG anti sheep IgG

Pig IgG anti sheep IgG was absorbed on a sheep IgG₁ and IgG₂ sepharose 4B column, after which it was eluted from the column.

Preparation of F(ab)₂ fragment of Pig IgG anti sheep IgG

Pig IgG anti sheep IgG was prepared as described above. It was mixed with pepsin (Sigma Chemical Ltd, Poole, England) in 0.07M Actate, 0.05 M NaCl buffer pH 4. Following incubation for 18 hours at 37°C the precipitate was removed by centrifugation. The pH of the supernatant was adjusted to 7.5 and the F(ab)₂ fragments obtained by separation on a G 100 column. The F(ab)₂ was affinity purified on a column of pig IgG anti sheep IgG (Fc) sepharose.

Horse radish peroxidase (HRP) conjugation

Aliquots containing 5mg of protein were conjugated with HRP (Type IV, Sigma Chemical Company, Poole, England) with the method described by Wilson and Nakane (1978).

SEROLOGY

Sera from both mice and sheep were tested for anti-toxoplasma antibodies by an adaptation of the ELISA first reported by Voller et al (1976)

Toxoplasma Antigen Preparation

Toxoplasma RH strain tachyzoites were obtained as previously described and washed three times in PBS by centrifugation at 2000 rpm for 15 minutes. After the final wash the supernatant was discarded and the pellet, which contained the tachyzoites, was lysed by resuspension in 10 times its volume of distilled water. The pellet was freeze-thawed three times and then sonicated for 60 seconds (MSE, Scientific Instruments, Crawley, England). Particulate cell debris was removed by centrifugation at 30,000 x g for 10 minutes. The supernatant was divided into aliquots and stored at -20°C until required. The protein concentration of the antigen preparation was estimated with a Pierce protein assay which uses a BCA working reagent (Pierce Chemical Company, Rockford, Illinois, USA) and approximately 2µg protein was added to each well in the test.

Krebs Tumour Antigen

In the sheep IgG-ELISA the ascitic fluid from a mouse tumour was used as a control antigen in an attempt to detect any non-specific binding to mouse components. This antigen was prepared in the same manner as the Toxoplasma antigen.

Concentrations of Reagents

The concentrations of the test sera and conjugates to be used in both the sheep and mouse ELISA were determined by chequer board titrations.

Protocol for the Sheep IgG-ELISA

Toxoplasma or Krebs tumour antigen was diluted with 0.05M carbonate/bicarbonate buffer pH9 to a predetermined concentration. 150 μ l of antigen solution was added to each well of a polystyrene microtitre plate (type 129A, Dynatech Laboratories Ltd., Billingham, England) with a multichannel pipette (Flow Laboratories, Irvine, Scotland) and the plates were incubated at 4°C overnight. Plates were then washed three times with PBS which contained 0.05 per cent Tween 20 (PBS/Tween), and the fluid removed after each wash. Test sera were diluted to 1/500 with one per cent BSA in PBS. Eight serial dilutions of a standard serum obtained from a sheep naturally infected with Toxoplasma were used in each test in order to construct a standard curve. Samples of 150 μ l of standard and test sera were added to duplicate wells and the plates incubated for two hours at 37°C and subsequently washed three times with the PBS/Tween solution. Each well was filled with 150 μ l of pig IgG anti-sheep IgG conjugated to horseradish peroxidase (type IV Sigma Company Ltd., Poole, England.) After incubation for two hours at 37°C the plates were emptied and washed.

The enzyme substrate solution was prepared by the addition of 4 mg of orthophenylenediamine (OPD) to 10 ml of phosphate-citrate buffer pH5 and 150 μ l added to each well. After 30 minutes at room temperature the reaction was stopped by the addition of 2.5M sulphuric acid. Results were recorded with a "Titertek multiskan"

multichannel spectrophotometer (Flow Laboratories, Irvine, Scotland.) set to measure optical densities at a calibrated wavelength of 492nm.

Protocol for the Mouse IgG-ELISA

The procedure for the mouse IgG-ELISA was similar to that of the sheep test, however the serum used for the standard curve was a pool obtained from experimentally infected mice. The test sera were used at a dilution of 1/200 whilst the rabbit IgG anti-mouse IgG peroxidase conjugate was diluted to 1/32000.

Calculation of Results

A curve Log 10 (Dilutions) vs O.D. was drawn with the results from the eight dilutions of standard serum and a corrected O.D. for the 1/500 dilution was obtained from this. The O.D. from the test sera were divided by this result and multiplied by 100 to obtain a "per cent O.D". Based on experience with the assay a positive result was taken to be any reading above 25 per cent with the sheep IgG-ELISA and any result above 15 per cent with the mouse IgG-ELISA.

An attempt was made to devise an indirect ELISA specific for anti-toxoplasma IgM, however the results obtained were inconsistent. Two possible reasons for this result are that competition was occurring between IgM and IgG in the test serum as has previously been reported (Filice et al, 1980). Alternatively the sheep anti-mouse IgM-HRP may have been of low affinity resulting in poor binding to the test sera.

CANNULATION OF POPLITEAL EFFERENT LYMPHATIC

Pre-operative procedures

Sheep were confined to metabolism crates, measuring approximately 100 cm x 200 cm x 120 cm, for several days before the operation to allow them to adjust to their environment.

Sheep were anaesthetised with a mixture of 50 per cent oxygen and 50 per cent nitrous oxide, including four per cent Halothane. Maintenance of this state was achieved with 2.5 per cent halothane in 50 per cent oxygen and 50 per cent nitrous oxide.

Procedure for Efferent Lymphatic Cannulation

The procedure first described by Hall and Morris (1962) was used. To aid the identification of the lymphatic vessel 0.25 ml of Evans blue dye (0.1 per cent in PBS) was injected s.c. into the drainage area of the popliteal lymph node.

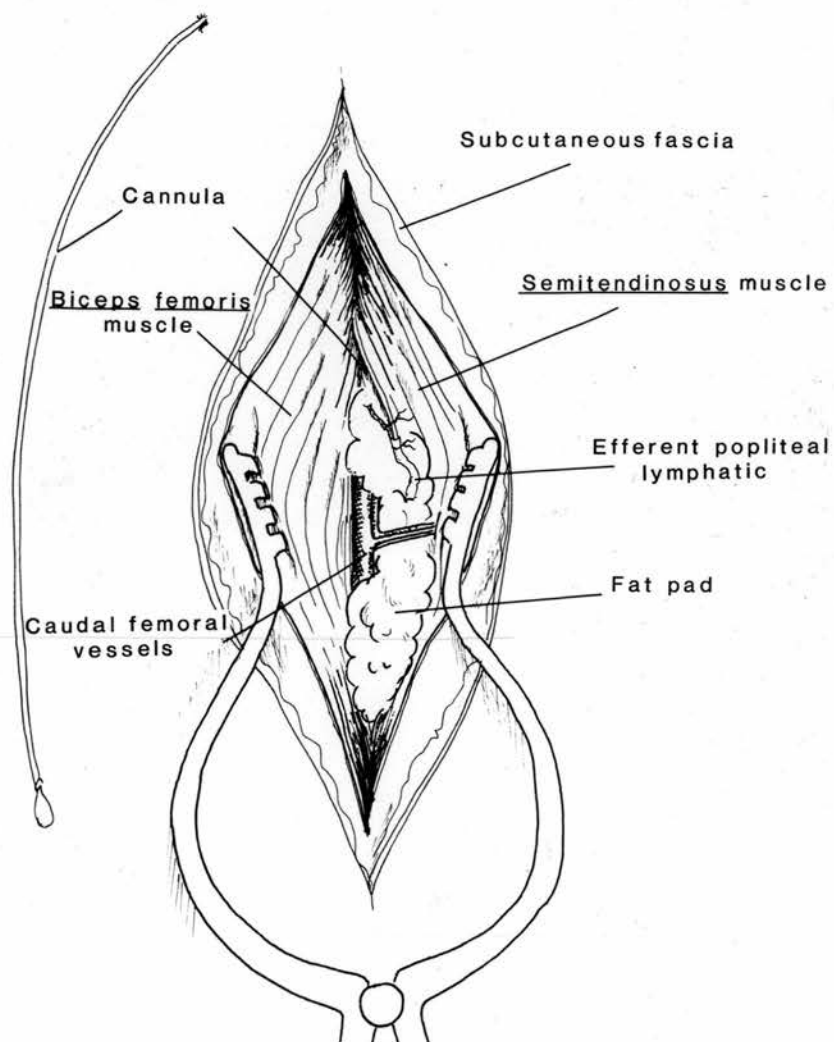
The skin and fascia was parted by an incision which started just below the tuber ischii and continued distally for approximately 15 cm over the groove between the semitendinosus and biceps femoris muscles (Fig 2.2). These muscles were separated and held apart with self retaining retractors. The pad of fat present in the groove was variable in size and crossed at its mid point by branches of the caudal femoris vessels and the sciatic nerve. Blunt dissection of this pad of fat exposed the efferent duct which ran cranially from the popliteal node, the position of which was variable and in many cases more than one was present. Sterilised polyvinyl tubing (Dural Plastics Engineering Ltd, New South Wales, Australia) was used as the cannula and was flushed with sterile physiological saline which contained 1000 international units of

heparin per ml. This was held in the tube by clamping one end with artery forceps. The other end was led into the operation site through a stab wound situated 5 cm above the upper limit of the original incision.

A section of the duct approximately 1 cm in length was isolated by blunt dissection and ligated with a silk ligature (Mersilk 5-0). A second ligature 1 cm distal to the first was laid around the duct, then an incision was made in the vessel between these ligatures with iris scissors. The cannula was inserted into the duct through this incision for a distance of 2-3 mm, and the distal ligature was tightened around it to hold it firmly in place. The clamp was then released from the opposite end of the cannula so that lymph flow could be verified. Following this the free ends of the proximal ligature were used to further secure the cannula in position.

The two layers of fascia were then repaired with 2-0 catgut, the fascia lata with simple interrupted sutures and the subcutaneous fascia with a continuous mattress suture. The skin was closed with interrupted vertical mattress sutures of monofilament nylon. In order that a collecting bottle could be secured to the animal, a rectangular piece of aluminium 5 cm x 2.5 cm x 2 mm, with two vertical slots, was sutured to the skin of the lateral aspect of the thigh anterior to and at the level of the proximal extremity of the skin incision. Collecting bottles were fastened to this by means of a length of umbilical tape which had been threaded through the slots in the plate.

FIGURE 2.2. Diagram of the position of the popliteal efferent lymphatic and surrounding tissues.



Post-operative care

Sheep were removed from the surgery and allowed to recover in metabolism crates which had been adjusted in size to measure 60 cm x 200 cm x 100 cm. Lymph was collected at least twice daily in plastic Falcon flasks which contained a small amount of powdered penicillin, streptomycin and heparin.

Cell count

Cell numbers in lymph were counted with a model ZBI Coulter counter (Coulter Electronics Ltd, Harpenden, England) fitted with a 100 μ m aperture tube. The instrument was set to count particles greater than 45 μ m.

Cytocentrifuge Preparations

Lymph cells were washed three times in HBSS and the concentration adjusted to 1×10^6 /ml in one per cent foetal calf serum (Gibco Ltd., Paisley, Scotland). Cytocentrifuge preparations (Shandon Ltd. Runcorn, England) were made on glass slides with 200 μ l of this suspension.

Differential Cell Counts

Representative cytocentrifuge preparation were stained with Leishman's solution and differential cell counts made.

Immunoglobulin Containing Cells

Selected cytocentrifuge preparations were fixed in two per cent paraformaldehyde in PBS for 10 minutes, and then stored until required in Tris saline buffer. In order to remove endogenous peroxidase slides were immersed in one per cent periodic acid for 10 minutes, washed with tap water and then placed in a one per cent solution of Na_2HPO_4 containing 0.2 per cent NaBH_4 for two minutes.

After two washes in 0.05 M Tris HCl buffer pH 7.6 either pig IgG anti sheep IgG or rabbit IgG anti sheep IgM were applied to the cytocentrifuge preparations for 90 minutes. Excess conjugate was removed by rinsing with 0.05M tris HCl buffer. The enzyme was visualised with 3'3-diaminobenzidine (4 mg in 10 ml) in tris buffer which contained 0.01 per cent hydrogen peroxide. The cytocentrifuge preparations were rinsed in running tapwater, counter stained with Meyer's haematoxylin, dehydrated and mounted.

SHEEP MAMMARY GLAND MACROPHAGES

Collection of Mammary Gland Macrophages

Macrophages were harvested according to the technique described by McDowell, Lee and Lascelles (1969). A normal saline suspension containing 5µg of lipopolysaccharide B (LPS) from E.coli serotype 0111 B4 (Sigma Chemical Company, Poole, England) was prepared. One teat and the surrounding udder was then swabbed clean with alcohol and the LPS suspension infused into the mammary sinuses via a blunt-ended 16 gauge needle. Washings from the mammary gland were obtained six days later by first infusing 10 ml of sterile saline into the gland, massaging the udder and then expressing the fluid into a sterile universal.

Medium

RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) was used throughout these experiments. Foetal calf serum (Gibco Ltd, Paisley, Scotland), 1.5M hepes, two per cent glutamine and one per cent pencillin and streptomycin were added to the medium prior to use.

Culture of Mammary Gland Macrophages

The cells collected by this method were washed three times in medium by centrifugation at 1100 rpm for 10 minutes. They were then counted with an Improved Neubauer chamber and their concentration adjusted to 1×10^6 per ml by the further addition of medium.

To enable efficient handling of samples, a chamber consisting of two multitest slides (Flow Laboratories, Irvine, Scotland) each with eight wells and two dividers was prepared (a unit). Each well was filled with 20 μ l of the cell suspension and the unit placed in a petri dish which contained moist tissues.

Cultures were maintained in an incubator (Flow Laboratories, Irvine, Scotland) in an oxygen/nitrogen atmosphere which contained five per cent CO_2 . After two hours incubation the non-adherent cells were removed from the wells by washing three times with medium.

Latex beads were added to some macrophage monolayers in order to check the phagocytic capacity of the cells. The macrophages monolayers were washed with PBS to remove any extracellular tachyzoites and then fixed with three per cent glutaraldehyde in 0.1 M phosphate buffer pH 7.4. They were then stained with Weigert's Haematoxylin.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate sampling buffer

The sampling buffer used consisted of 25 per cent stacking buffer, four per cent ^{SDS} 20 per cent glycerol, 10 per cent mercaptoethanol and 0.002 per cent bromophenol blue.

Toxoplasma Antigen preparation.

Toxoplasma RH strain tachyzoites were prepared as previously described in this chapter. They were washed three times in PBS and centrifuged at 800g for 25 minutes. The pellet was then suspended in SDS sampling buffer and boiled for 6 minutes.

Toxoplasma antigens were separated by discontinuous SDS electrophoresis in polyacrylamide gels according to the procedures described by Lamelli (1970). The stacking and resolving gels contained three and 10 per cent acrylamide respectively and the dimensions of the gel were 140 mm x 160 mm x 0.75 mm.

The Toxoplasma preparation was added into a single large well which resulted in a wide band of separated proteins which after transfer could be processed in strips. Gels were loaded with the Toxoplasma preparations at a protein concentration of 5µg/mm²DU. A single track of molecular weight marker proteins was included in each run, they were as follows:

Equine cytochrome C	12.3 kD
Equine myoglobin	17.2 kD
Bovine chymotrypsinogen	25.7 kD
Hen egg ovalbumen	45 kD
Bovine serum albumin	66.25 kD
Hen egg ovatransferrin	76-78 KD

Electrophoresis was carried out under constant current conditions at a current density of 19m A/cm² until the tracking dye (bromophenol blue, 0.001 per cent) included in the sample buffer had migrated approximately 9 cm into the resolving gel (2-2.5 hours).

Transfer of separated proteins to nitro cellulose membrane

The separated proteins were transferred from the polyacrylamide gel to nitrocellulose membranes by means of a semidry electroblotter (produced at M.R.I.). This semidry electroblotter consists of two separate graphite sheet supported by perspex plates. A sandwich which contains the polyacrylamide gel and nitrocellulose membrane is placed between the plates and a current of 200 mA passed through for one hour. The sandwich was prepared by soaking pieces of Whatman 3 mm (Whatman Ltd., Maidstone, UK) filter paper cut to the size of the gel in various buffers. Two sheets of filter paper were soaked in 0.3 M Tris which contained 20 per cent methanol, and these were placed on the lower graphite plate (the anode). A "unit" was then constructed which consisted of one sheet of filter paper soaked in 25 mM Tris with 20 percent methanol, a sheet of nitrocellulose membrane, pore size 0.2 (Schleicher and Schull, Dassel, West Germany) followed by the polyacrylamide gel. Two more sheets of filter paper soaked in 25 mM tris buffer which contained 40 mM 6-amino-n-hexanoic acid and 20 per cent methanol were then placed on top.

Immunodetection procedures

The wash buffer (WB) used throughout these procedures was 8.7 mM sodium phosphate buffer pH 7.2 which contained 500 mM NaCl, 0.5 per cent "Tween 80" and 1.0 mM EDTA. During all the procedures the membrane was agitated gently on a rotary shaker.

Strips approximately 5 mm wide were cut from the nitrocellulose membrane. The non-specific binding sites in the membrane were "blanked" by incubating with 10 per cent ovalbumen in WB for one hour at 37° C. The strips were then transferred to

individual plastic tubes which contained the test sera (1 in 40 dilution in WB with five per cent ovalbumen) and incubated for one hour at room temperature. The strips were washed thoroughly after the incubation by three rapid rinses, three 5 minutes immersions and three more rapid rinses in WB.

Strips were then transferred to the detection serum which was either affinity-purified rabbit IgG anti-sheep F(ab)₂ (prepared at MRI) for the ovine sera or Protein A purified from *S. aureus* Cowan strain (Sigma Chemical Ltd, Poole, England) for the murine sera. These were labelled with ¹²⁵I (Amersham International UK, Amersham, England) by the chloramine T reaction (Hunter and Greenwood, 1962) and used at previously determined optimal dilutions. After an incubation period of one hour at room temperature the strips were given a thorough wash and dried under vacuum. They were mounted on filter paper before being exposed to X-ray film (Kodak X-Omat, Kodak, Liverpool, England) in a radiographic cassette which contained an intensifying screen. The X-ray film was exposed overnight at -70° C and then developed in D19 Developer (Kodak, Liverpool, England) and subsequently fixed in Kodafix (Kodak, Liverpool, England).

Coomassie blue staining of polyacrylamide gel

After electrophoresis the gel was stained in 0.1 per cent Coomassie blue R250 (Sigma Ltd, Poole, England) which contained one per cent trichloroacetic acid, 7.5 per cent acetic acid and 50 per cent methanol for 1/2 - 1 hour. Destaining was carried out as required and was achieved by immersion in the above solution minus the dye.

Comassie blue staining of nitrocellulose membrane

To permit detection of the molecular weight standards and the array of proteins transferred, the nitrocellulose membrane was stained for 30 minutes in a solution of 30 per cent methanol and five per cent acetic acid with 16 μ g/ml of Coomassie blue. Destaining if required was achieved by the addition of the same solution without the dye.

RECTAL TEMPERATURES

Rectal temperatures of sheep were recorded with an electronic thermometer (Digitron Instrumentation Ltd, Hertfordshire, England).

LAMBS WEIGHTS

Lambs were weighed with a mechanical balance.

FIXATION AND PROCESSING OF TISSUE FOR HISTOLOGICAL STAINING

Brains and cotyledons from experimental animals were fixed by immersion in 10 per cent formol saline for a minimum of one week. Coronal slices of brain made up of three slices from cerebrum, anterior, mid and posterior, one slice through mid brain and one through cerebellum with a sagittal section through the cerebellar vermis. Blocks of tissue were also cut from placental cotyledons. These selected tissues were then post fixed in modified Bouin (saturated aqueous picric acid, 190 ml; 40 per cent formalin; 10 ml, glacial acetic acid; 5 ml) for two hours and processed through graded alcohols, toluene and polywax. Sections 4 μ m thick were cut and stained with Mayer's haematoxylin and eosin (HE) for light microscopy.

Cross-sections of ovine popliteal and prescapular lymph nodes were taken and further subdivided to give blocks approximately 3mm. These were allowed to fix for two hours at 4°C in modified Bouin's fixative then dehydrated through graded alcohols at 4°C, cleared in xylene and embedded in paraffin wax. Sections 4µm thick were cut, dewaxed in xylene, rinsed in ethanol and placed in one per cent hydrogen peroxide in methanol to remove endogenous peroxidase. They were then washed in 0.01M PBS which contained two per cent egg albumen. Rabbit IgG anti-sheep IgM and pig IgG anti-sheep IgG peroxidase conjugates were applied to separate sections for one hour. Excess conjugate was removed by rinsing the sections with PBS containing albumen followed by 0.05M tris HCl buffer pH 7.6. Enzyme was visualised as previously described. The sections were then rinsed in running tap water, counterstained with Mayer's haematoxylin, dehydrated and mounted.

CHAPTER 3 Titration of Toxoplasma gondii M1 strain oocysts in mice.

INTRODUCTION

In order to obtain a stock of T.gondii oocysts for use in both ovine and murine experiments, a total of four cats were fed mouse brain which contained Toxoplasma tissue cysts (Chapter 2). Verification was required that the oocysts shed in the cats' faeces belonged to the genus T.gondii and not to another coccidium and that they were infectious to mammals.

EXPERIMENTAL PROCEDURE

The concentration of sporulated oocysts (Fig. 3.1) was adjusted to 5×10^3 per ml in HBSS. Doubling dilutions of this suspension were prepared and each dose suspended in 0.2 ml, was inoculated i.p. into four mice. This procedure was repeated three times with oocysts obtained from two different cats, therefore a total of 12 mice received each treatment (Table 3.1). Four weeks after infection the mice were killed and blood and brains collected. Sera was removed following centrifugation of clotted blood and was tested in the mouse IgG ELISA (Chapter 2). Each brain was placed in a universal bottle which contained 1 ml PBS plus one per cent pen/strep and left overnight at 4°C. After homogenisation (Chapter 2), five 5µl smears were made and stained with Giemsa for five minutes. The remainder of each of the brain homogenates was diluted 10 times with PBS and 1.5 ml of 30 per cent Percoll layered below it. A second layer, (1.5 ml) of 90 per cent Percoll was then introduced below the 30 per cent layer. Samples were centrifuged for 15 minutes at 3,500g after which the debris formed a mat above the 30 per cent Percoll. The solution below this layer, where the

tissue cysts were considered to be, was collected with a pasteur pipette. Samples were examined in a McMaster worm egg counting slide.

The length and width of 50 oocysts, 50 tissue cysts in Giemsa stained smears (Fig. 3.2) and 50 tissue cysts separated by the Percoll technique (Fig. 3.3) were measured microscopically with an image shearing eyepiece (Vicker's Ltd). In order to obtain a digital readout of these dimensions the microscope was linked to a digitiser.

RESULTS

Detection of tissue cysts in infected mouse brain

Out of the 108 experimental mice used, 10 died before the four week incubation period had ended and they were excluded from the results.

No tissue cysts were detected in the brains of mice that received 15 or less oocysts, irrespective of the counting technique used (Fig. 3.4 and 3.5). In the groups inoculated with higher doses of oocysts a minimum of five mouse brains per dose level contained tissue cysts. Tissue cysts were found more frequently and in higher numbers in Giemsa stained smears than in the Percoll preparations (Table 3.2 and Fig. 3.4, 3.5).

Detection of anti-toxoplasma IgG antibody

The mean per cent O.D. for each group from the three titrations is recorded in figure 3.6. No anti-toxoplasma antibody was detected in mice that received seven or fewer oocysts and of the animals which received 15 oocysts only one mouse was positive in

the mouse IgG ELISA. A comparison of the tissue cyst results and the serological data is shown in table 3.3. Although the results correlate well it is apparent that a small number of animals appear positive with one test and negative in the other.

Dimensions of Oocysts and Tissue Cysts

The fifty oocysts^{which} were measured had mean dimensions $13.0\mu\text{m} \pm 0.3\mu\text{m} \times 10.3\mu\text{m} \pm 0.9\mu\text{m}$. The average size of the tissue cysts obtained by Percoll separation was $23.1 \pm 4.1\mu\text{m} \times 23.6 \pm 4.2\mu\text{m}$ and those in the stained smear preparation were similar ($22.8 \pm 1.1 \times 23.2 \pm 12.1\mu\text{m}$).

DISCUSSION

This study was designed to determine the identity and infectivity of the oocysts obtained from the experimentally infected cats and in addition to compare two methods of counting T.gondii tissue cysts.

The i.p. route was used for the mouse inoculations since it has previously been demonstrated to be more sensitive than oral dosing for the detection of oocysts in a sample (Dubey and Frenkel, 1973). Four weeks after the inoculation of oocysts both Toxoplasma tissue cysts and anti-toxoplasma IgG antibody could be detected.

Together these facts indicate that the oocysts shed in the cat faeces were those of T.gondii. The figures obtained for the dimensions of the sporulated oocysts ($13 \times 9\mu\text{m}$) compare well with those previously published for T.gondii (Dubey, 1977).

The number of brains found to contain tissue cysts was greater when stained smears were used rather than the Percoll separation technique. In addition larger numbers of tissue cysts were detected when the former technique was used. This would seem to indicate that efficiency of the extraction process was not optimal, perhaps because the cellular debris present after the centrifugation step trapped a proportion of the tissue cysts. The skill of the operator at performing the Percoll separation or at identifying tissue cysts in a brain homogenate may also play a role in determining the numbers of tissue cysts counted, since Blewett and coworkers found more tissue cysts with the former technique (Blewett, Miller and Harding, 1983).

The mouse IgG ELISA was slightly more sensitive at detecting Toxoplasma infection in mice compared with the identification of tissue cysts in mouse brain. Furthermore this test is less time consuming than the examination of mouse brains by light microscopy and the operator does not require the same degree of skill. However since the sera of six mice, which had tissue cysts in their brains, contained no detectable anti-toxoplasma IgG, it may be advantageous to use both techniques for the diagnosis of Toxoplasma infection in mice.

CONCLUSIONS

The data reported here indicate that the T.gondii oocysts excreted in the faeces of the experimentally infected cats were infectious for mice. In addition it is suggested that both serological tests and microscopic examination of the brain should be performed in order to achieve an accurate diagnosis of infection in mice.

TABLE 3.2: Comparison of Percoll separation technique with Giemsa stained brain smears.

		PERCOLL	
		POSITIVE	NEGATIVE
GIEMSA STAINED SMEARS	POSITIVE	28	10
	NEGATIVE	3	57

TABLE 3.3: Comparison of microscopic examination of brain for tissue cysts with serological examination.

		TISSUE CYSTS	
		POSITIVE	NEGATIVE
IgG ANTIBODY	POSITIVE	36	5
	NEGATIVE	6	51

FIGURE 3.1 Toxoplasma gondii sporulated oocysts
isolated from cat faeces and allowed
to sporulate at 22°C for 48 hours.
Oocysts examined by phase contrast.

X 400

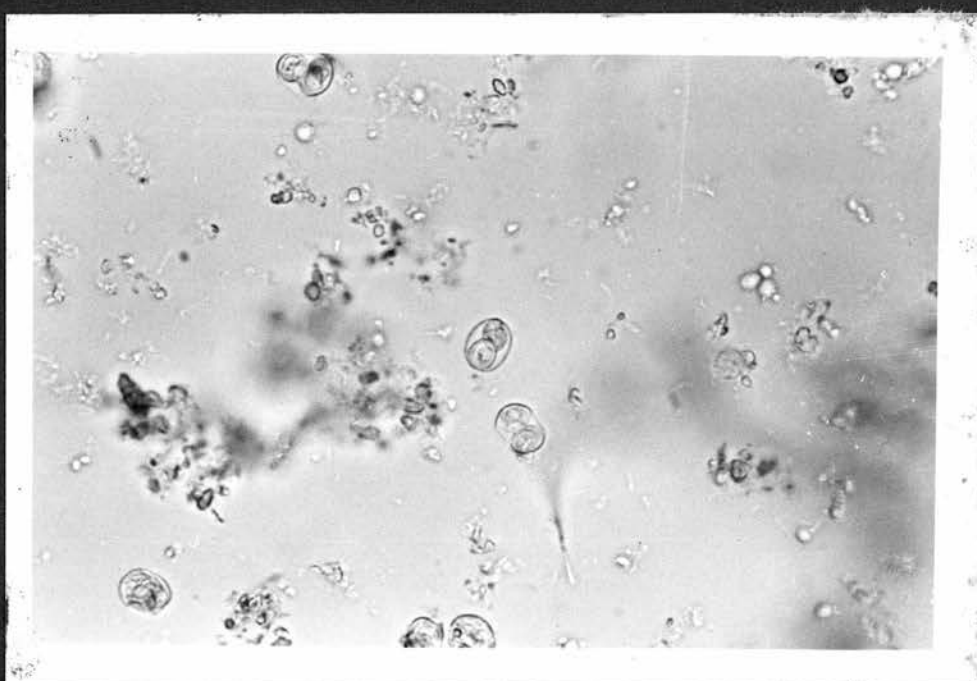


FIGURE 3.2 Toxoplasma gondii tissue cyst in a
smear of infected mouse brain which was
stained by Giemsa.

X 400

FIGURE 3.3 Toxoplasma gondii tissue cysts
obtained from infected mouse brain
by Percoll separation and viewed by
phase contrast.

X 200

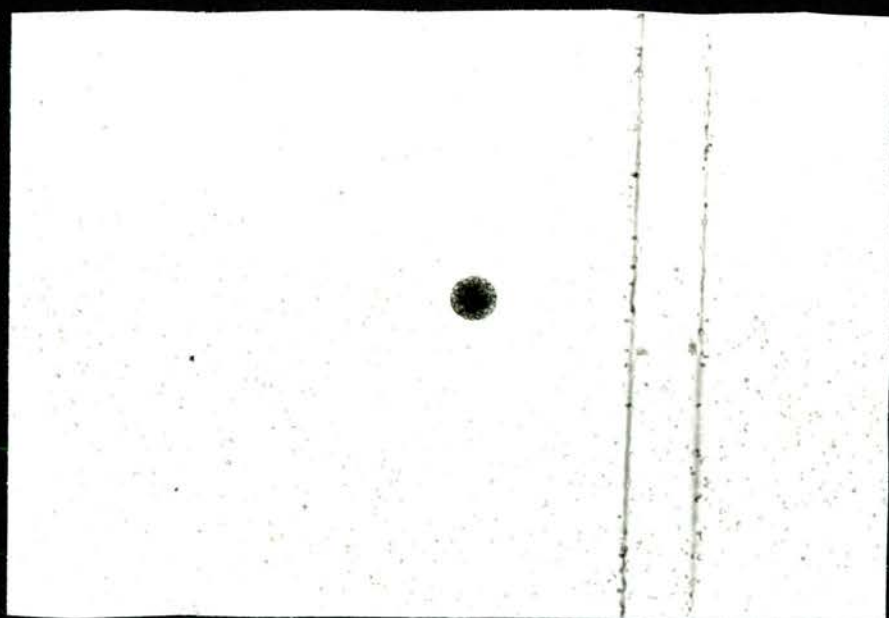
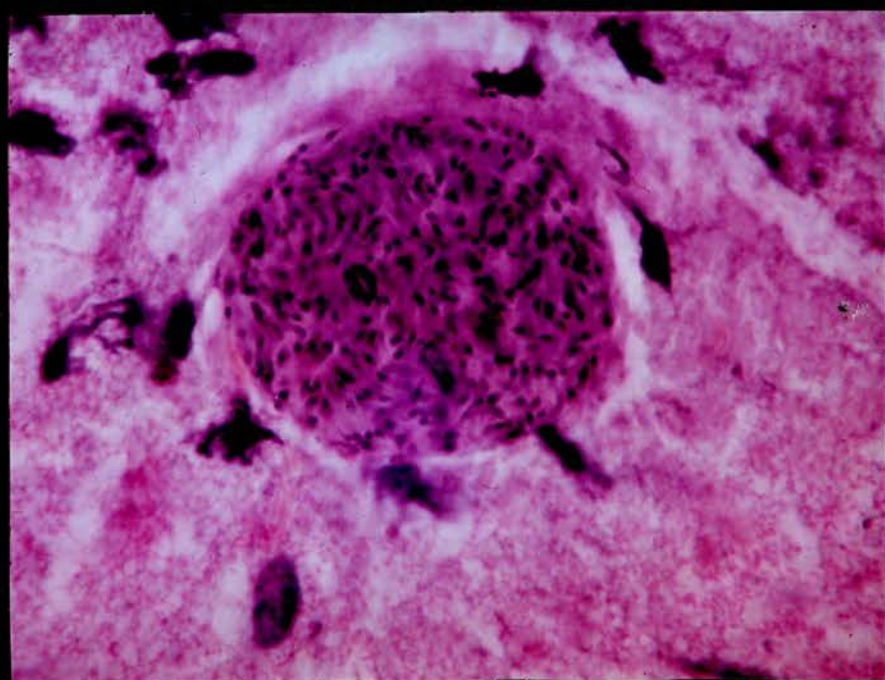


FIGURE 3.4 Mean number of Toxoplasma gondii
tissue cysts detected by microscopic
examination of Giemsa stained
preparations of homogenised mouse
brains.

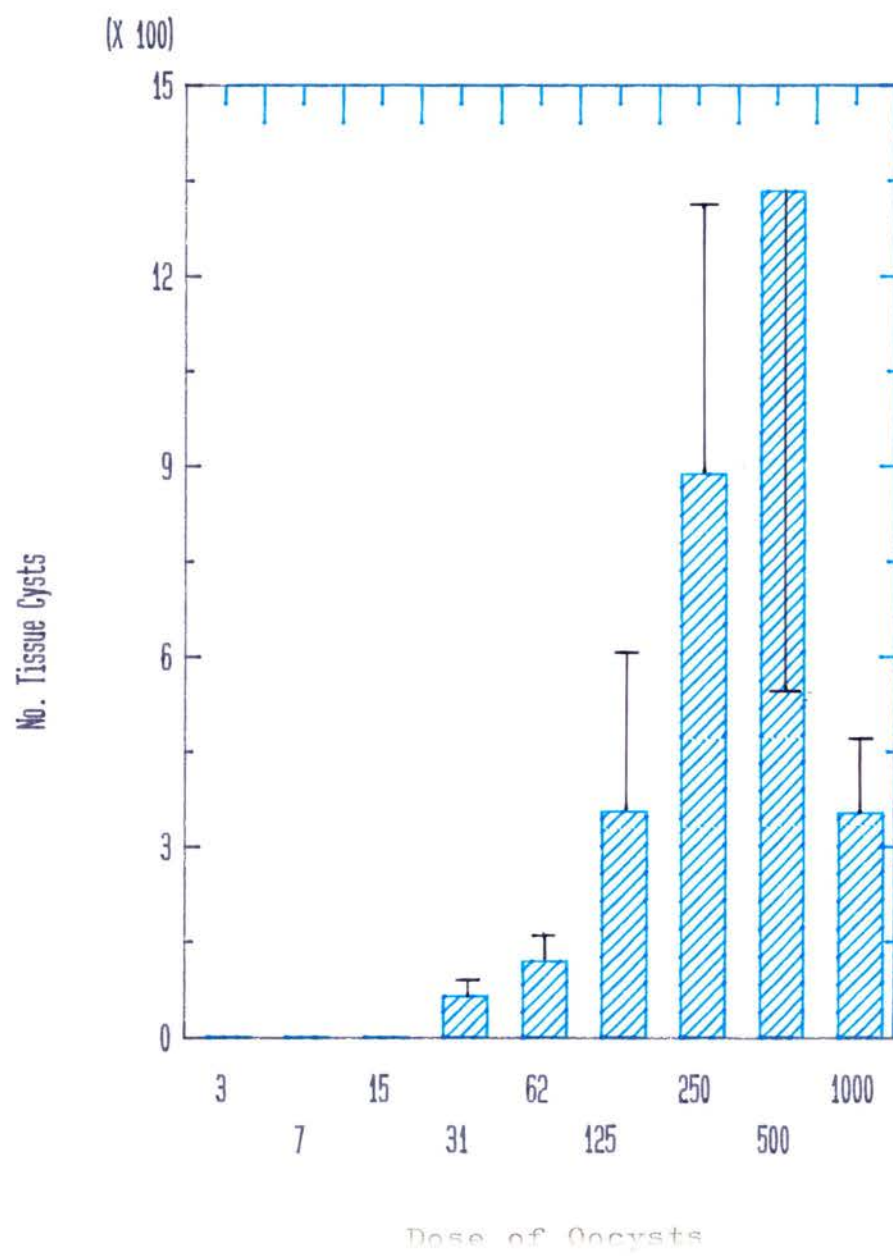


FIGURE 3.5 Mean number of Toxoplasma gondii
tissue cysts detected by microscopic
examination of Percoll separations of
homogenised mouse brains.

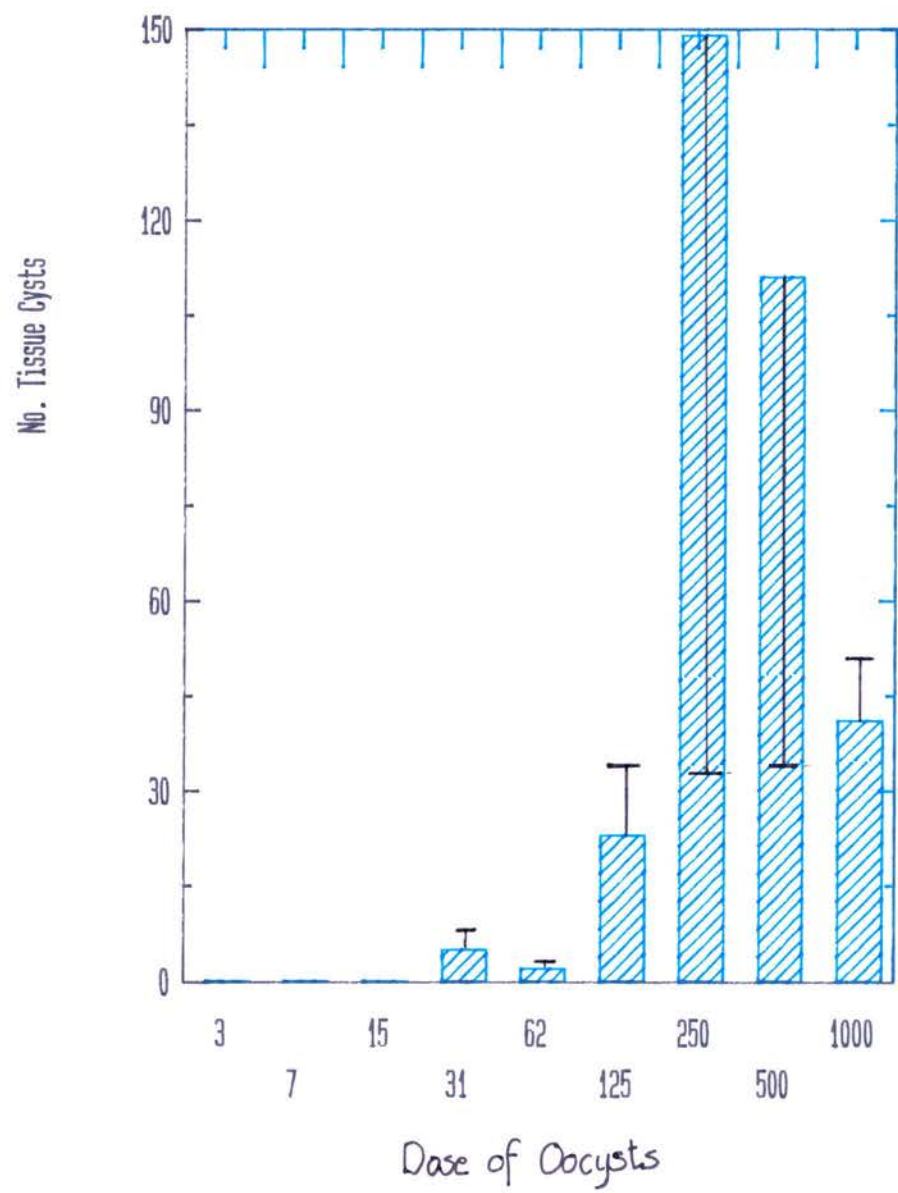
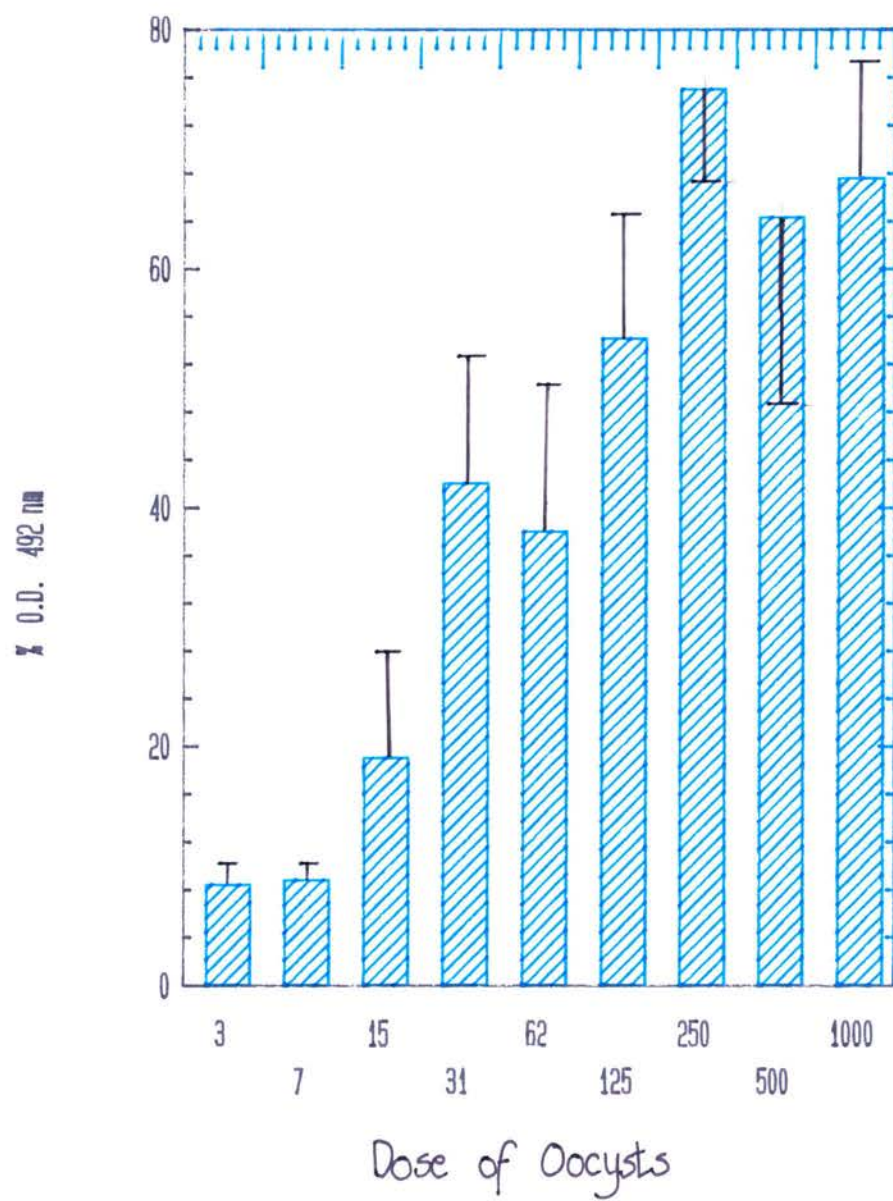


FIGURE 3.6 IgG against Toxoplasma gondii detected
by mouse IgG ELISA (mean per cent O.D).



CHAPTER 4 Titration of Toxoplasma gondii M1 strain oocysts in
non-pregnant sheep and the effects of subsequent
challenge of these animals during mid-gestation.

INTRODUCTION

Ingestion of T.gondii oocysts is the most plausible mode of transmission of the parasite to sheep and several reports from natural outbreaks of clinical toxoplasmosis have provided good circumstantial evidence that oocysts were the source of the infection (Plant et al, 1974; Dubey et al, 1986). The first reported experimental infection in sheep with oocysts was in 1975, when eight sheep were fed 10,000 sporulated oocysts during mid pregnancy and all of them seroconverted and later aborted (Beverley et al, 1975). Since then several other workers have used T.gondii oocysts, although most studies have continued to use tissue cysts, perhaps because they are more readily obtained than oocysts. Dubey and Sharma (1980) successfully isolated Toxoplasma from the tissues of animals orally infected with either 100 or 100,000 GT-1 oocysts. Subsequently it has been shown that animals given an oral dose of 10,000 oocysts developed a pyrexia (Dubey, 1984). However no serological data was reported in these studies and the four animals inoculated with 100 oocysts were not monitored for clinical symptoms. More recently it has been shown that all ewes inoculated orally with 2000 M1 strain oocysts developed a pyrexia and subsequently anti-toxoplasma antibody. Furthermore vertical transmission of the infection occurred in every case (Buxton, Blewett, Trees, McColgan and Finlayson, in preparation).

However there is no information on the minimum number of oocysts required to cause infection in sheep as the parasite has never been titrated in these animals, with most workers having used very large numbers of oocysts. One study carried out in pigs demonstrated that when gilts were fed between four and 150 oocysts,

no clinical signs were apparent, although all the animals seroconverted (Durfee, Ma, Wang and Cross, 1974).

It is important from an epidemiological standpoint to define the number of oocysts required to cause infection. It is also of value to determine if seroconversion a) accompanies clinical symptoms or if they can occur independently and b) if seroconversion is indicative of a protective immune response as judged by a challenge infection. The aim of the study was to answer these questions.

EXPERIMENTAL PROCEDURE

T.gondii oocysts obtained as previously described (Chapter 2), were counted in an improved Neubauer counting chamber and diluted with HBSS which contained one per cent pen/strep, to the required concentration. In the case of the titration experiment 10-fold dilutions of this oocyst suspension were made.

Fifty-nine Scottish Blackface ewes which had no detectable Toxoplasma antibody by IgG-ELISA were employed in this experiment. Animals in four treatment groups were orally inoculated 56 days before their mating. Nineteen animals received 2000 oocysts (group 1) whilst 10 ewes were given 200 (group 2) and a further 11 received 20 oocysts (group 3). There were 19 control animals which were inoculated with HBSS only (Table 4.1).

Ewes were synchronised for oestrus and served by Suffolk rams. Animals which did not hold to the first service were returned to the ram at the beginning of their second cycle. At approximately 70 days gestation the ewes were scanned by ultrasound for the presence of fetuses. Six animals were found to be non-pregnant

and together with another two which had not been served they were removed from the experiment.

The remaining ewes were given an oral challenge of 10,000 oocysts or HBSS between 77 and 83 days of gestation. All the animals which had initially received 200 or 20 oocysts (groups 2 and 3 respectively) were challenged along with eight ewes from group 1 (2000 oocysts) and 11 previously uninfected animals (group 4A). The remainder of the ewes in the last two groups received no further treatment (groups 1A and 4B).

After inoculation, rectal temperatures were recorded from day 4 until either day 11 or 14 after infection. The IgG-ELISA and, on selected occasions, the IHA test were used to detect anti-toxoplasma antibody present in fortnightly serum samples.

Ewes were monitored closely during the lambing period and the placentae from all ewes and brains from all aborted and stillborn lambs were collected for histopathology. In addition a blood sample was collected from live and stillborn lambs and the serum from this, separated and stored at -20°C until examined for anti-toxoplasma antibody with the IgG-ELISA. All live lambs were weighed at birth. Impression smears of placental cotyledons were made, stained with a modified Ziehl-Neelsen's and examined for the presence of Chlamydia psittaci organisms. Statistics quoted for temperature and antibody data were obtained by analysis of variance.

RESULTS

TITRATION OF OOCYSTS

Temperature response

Daily mean rectal temperatures from each group are recorded in

figure 4.1, a reading of greater than 40°C was defined as a febrile response. All animals which received 2000 oocysts (group 1) developed a pyrexia which lasted on average from day 6 to day 10 after infection and these temperatures were significantly higher than those in the other groups ($P < 0.001$). Whilst the mean temperature in animals which were inoculated with 200 oocysts (group 2) rose slightly between seven and 10 days after inoculation at no time was it greater than 40°C and neither was it significantly different from either animals inoculated with 20 oocysts or control ewes which all remained afebrile. Two animals from group 2 developed a characteristic febrile response whilst the other eight remained clinically normal (Fig. 4.2).

Serology

In order to compare the IgG ELISA with a more established test 140 serial serum samples from all the experimental groups were tested by both the IgG ELISA and the IHA test (VI centre Edinburgh). The data was compared by regression analysis and a linear relationship was found, the correlation coefficient being 0.873.

IgG antibody response

No anti-toxoplasma IgG was detected in either control animals or those which received 20 oocysts, however, it was detected in animals inoculated with 2000 oocysts (group 1) 14 days after infection and by week 4 this was significantly increased compared to all the other groups ($P < 0.001$) (Fig. 4.3). The amount of specific antibody continued to rise in group 1 and reached a plateau at week 8. However at 16 weeks after inoculation a further increase in specific IgG was detected. The mean antibody response

in ewes that received 200 oocysts (group 2) showed a slight increase by week 4 and further dissection of this response (Fig 4.4) revealed that three ewes, only two of which had become pyrexia following inoculation had seroconverted while the other seven had not developed any detectable anti-toxoplasma antibody.

CHALLENGE EXPERIMENT

Temperature Response

Animals that received the 10,000 oocysts alone ("nil/10,000") and those inoculated with 20 oocysts and subsequently challenged ("20/10,000") (groups 4 and 3 respectively) showed a febrile response which persisted for four days beginning on day 4 after infection (Fig 4.5). The initial non-responders from group 2, which had been inoculated with 200 oocysts also developed a pyrexia upon challenge, whereas the two responder sheep remained clinically normal (Fig. 4.6). All animals which had been infected with 2000 oocysts during the titration remained clinically normal whether or not they received the challenge inoculum (groups 1A and 1B). Furthermore none of the control animals became febrile (group 4B). The temperatures in groups 2, 3 and 4A were significantly higher than groups 1A, 1B and 4B from day 5 to day 8 after infection (At the peak of the response group 2 vs groups 1A or 1B, $P < 0.001$; vs group 4B, $P < 0.01$; groups 3 or 4 vs groups 1A, 1B or 4B, $P < 0.01$). No other clinical signs of illness were detected in any of the sheep.

Ig G Antibody Response

At the time of challenge detectable antibody to T. gondii in animals given 2000 oocysts in group 1 was high and no difference was apparent after the challenge (group 1B) (Fig. 4.7). All

animals inoculated with 200 and then 10,000 oocysts (group 2) had developed anti-toxoplasma antibody by the second week after challenge. The presence of specific IgG was first detected in "20/10,000" and "nil/10,000" animals one week after inoculation and this rose steadily until the last sample was collected seven weeks post infection. Control ewes not challenged with infective inoculum (group 4B) showed a mean increase in antibody three weeks after the challenge infection had been given to the other animals and this can be explained by one ewe seroconverting. However the level of specific antibody in the challenge groups were significantly higher than in the control group ($P < 0.001$).

LAMBING

Sixty nine lambs and foetuses out of a possible 74 were collected. Although signs of abortion were normally clear, two sheep, one from the "200/10,000" oocysts group expecting twins and one from the "20/10,000" group expecting a single gave no indications of having aborted. The predictions from the scanning were correct for all live and late abortions and it is assumed that these two ewes aborted early and that their foetuses were lost.

Live Lambs

The lambing results are recorded in table 4.2, and are expressed both as the number of ewes giving birth to at least one live lamb and as the total number of live lambs born in order to account for twins.

The seven ewes which received only 2000 oocysts produced 10 live lambs whereas the eight animals inoculated with "2000/10,000" oocysts gave birth to 10 out of a possible 11. In group 2

("200/10,000") six lambs were delivered by four ewes while in the group which received 20 oocysts and were then challenged only two ewes produced a single lamb each. Control animals (group 4B) delivered seven live lambs and three ewes from group 4A ("nil/10,000") gave birth to four out of a possible 15 lambs.

Statistical analysis of the proportions of live lambs in each group by Fisher's 2-tailed test revealed that groups 1A, B and 4B had significantly more lambs than any of the other three groups. (Group 1A vs group 2, $P < 0.01$; vs groups 3 or 4A, $P < 0.001$. Group 1B vs group 2 $P < 0.05$; vs group 3, $P < 0.001$; vs group 4A, $P < 0.01$. Group 4B vs group 2, $P < 0.05$; vs groups 3 or 4A, $P < 0.01$).

At lambing four ewes from groups 1A and 1B gave birth to classical border disease (BD) infected lambs, which were easily recognisable as "hairy shakers". Monthly serial serum samples from November to February were tested by a neutralisation assay (Gardiner, Nettleton and Barlow, 1983) to determine if and when the ewes had seroconverted. Ten out of the 59 ewes had antibody to BD virus and of these six had been infected prior to service. Although two of these were barren the remainder gave birth to normal live lambs. The remaining four ewes had seroconverted between 23 and 65 day of gestation, suggesting that infection had occurred during early pregnancy and whilst one of these was barren, two had classical hairy shaker lambs and one produced a stillbirth. Histopathological examination of this lamb's brain revealed the characteristic demyelination found in BD (Barlow, 1982). One other ewe which was serologically negative on the last day tested also gave birth to two BD virus infected lambs, it is assumed that the ewe had been infected around 60 days gestation and would not have

produced specific antibody by the last testing date but could have given birth to clinically infected lambs.

All the infected ewes had been housed in the same pen prior to and immediately after mating and this was adjacent to a pen containing ewes experimentally infected with BD virus. However the entry to the sheep house and the passages were shared and sheep from my experiments were routinely bled in the passage way.

Toxoplasma Infection- Histopathology of the Cotyledons and Serology

The cotyledons collected from the ewes were fixed, stained (Chapter 2) and then examined for focal necrosis (Fig 4.8 and 4.9). Cotyledons from control animals (group 4B) and those that received 2000 oocysts during the titration (groups 1A and 1B) showed no signs of Toxoplasma infection, whereas focal necrosis was seen in the cotyledons from all animals in the treatment groups "nil/10,000" oocysts and "20/10,000" oocysts (Table 4.3). Two of the three placentae examined from animals which received 200 oocysts and the challenge inoculum had focal necrosis in their cotyledons.

Specific IgG antibody in precolostral sera was tested for with the IgG-ELISA (Table 4.3.). All ewes from group 4B and those which had initially been given 2000 oocysts (groups 1A and 1B) gave birth to lambs which were seronegative to Toxoplasma except for one lamb produced by a control ewe which was seropositive. Specific IgG antibody was detected in this lamb's mother three weeks after the challenge infection had been administered to the other animals. No placenta was recovered and so this serological finding cannot be confirmed by histopathology.

Precolostral sera from live lambs in the groups given "20/10,000" or "nil/10,000" oocysts all contained anti-toxoplasma antibody. Five out of the six sera collected from group 2 lambs "(200/10,000 oocysts)" gave positive results with the IgG-ELISA whilst the lamb which had no detectable anti-toxoplasma antibody also had no detectable lesions in its placental cotyledons.

Weights of Lambs

The average weights of the live lambs in each group are recorded in table 4.4. The lambs which were congenitally infected with BD have been removed from this analysis. The original figures have also been adjusted to take into account the presence of twins. The mean weight of lambs in groups receiving "2000/10,000" or 2000 oocysts only was more than 4.5 kg. Those lambs born to animals initially inoculated with 200, 20 or no oocysts and then challenged (Groups 2,3 and 4a respectively) had average weights less than 4 kg whilst the lambs from the control group were more than 4.5 kg. Comparison of the mean weights from groups 1A, 1B and 4B with the other three groups by analysis of variance showed that these weights were significantly higher ($P < 0.001$) whether or not the figures were adjusted for the effect of twins.

Dead lambs

The proportion of dead lambs produced in each group are shown in table 4.2. These figures are divided into those lambs which aborted soon after the challenge infection was given, either during or just after the pyrexia and those which aborted between 119 -143 days of gestation. The reasons for this will become apparent later. There was one stillborn lamb from the group of ewes which received "2000/10,000" oocysts whereas those animals which were

inoculated with 200 oocysts and then challenged produced nine dead fetuses. Seven early abortions and four still births were collected from animals inoculated with "20/10,000" oocysts while those that received only the challenge inoculum aborted four fetuses soon after inoculation and another six close to the expected time of parturition.

Toxoplasma infection Serology and Histology of Brains and Cotyledons

The one dead lamb from group 1B "(2000/10,000 oocysts)" was negative for Toxoplasma infection both by histopathology of the brain and cotyledons and by serology. All the brains examined from groups that received 200, 20, or no oocysts and the challenge inoculum, had leucomalacia and / or focal inflammation, characteristic of Toxoplasma infection (Fig. 4.10 and 4.11, Table 4.5). Examination of placental cotyledons revealed several which had no focal necrosis, however they were all derived from fetuses which had been aborted early following the pyrexia. Due to the condition of the fetuses at abortion very few sera were obtained from lambs in groups given "200/10,000," "20/10,000" or "nil/10,000" oocysts (groups 2, 3 and 4A respectively) and although the sera from groups 2 and 3 were all positive, in group 4A there were three negative sera (Table 4.5). No evidence of chlamydial infection was found in sections or smears of cotyledons.

DISCUSSION

As the present literature on ovine toxoplasmosis contains very little work concerning experimental oocyst infection, the experiment described here was designed to increase the serological and clinical information available.

From recent work at the Moredun Research Institute (MRI) it is known that ewes can be orally infected with 2000 M1 strain oocysts and if dosed during mid-pregnancy vertical transmission will occur (Buxton et al, in preparation). With these results in mind the first part of the experiment involved a simple titration of oocysts in non-pregnant ewes. The second part involved a challenge of pregnant ewes in mid gestation with a high dose of oocysts. Both clinical symptoms and serology were monitored throughout the experiment.

TITRATION

All the animals which received an initial dose of 2000 T.gondii oocysts developed a pyrexia by day 6 which lasted for an average of five days. By day 14 after infection these animals had detectable levels of anti-toxoplasma IgG antibody. Both the control animals (group 4) and those given 20 oocysts (group 3) remained clinically normal throughout this period and specific antibody could not be detected. The data obtained from animals inoculated with 200 oocysts was not as clear cut, with three animals seroconverting and two of these also developing a febrile response, whilst the other seven ewes showed no such signs of infection.

A febrile response following infection with T.gondii has been reported by several workers (Smith, 1961; Miller et al, 1982; Buxton and Finlayson, 1986). The onset and duration of this pyrexia varies depending on the route of infection, form of Toxoplasma and dose but it normally becomes apparent four to six days after infection. Pyrexia at this stage coincides with the acute phase of the infection and with a transient parasitaemia (Jacobs and Hartley, 1964; Reid et al, 1982) and terminates with

the appearance of specific antibody (Miller et al, 1982), it has therefore been suggested that it is the development of an immun^ological response that suppresses the acute phase and stops the parasitaemia.

From the data it appears that 200 Ml strain oocysts given orally is the minimum number of oocysts which is capable of inducing an active infection in sheep, since only a proportion of ewes given this dose underwent a febrile response and seroconverted. Why the required dose is larger than in pigs (Durfee et al, 1974) may in part be explained by anatomical differences in the gastro-intestinal systems of the two species and it is possible that some "dilution" may have occurred in the fore stomachs of the animals used in this study. Why only 30 per cent of ewes became infected as defined by an antibody response is not known. However several factors may be involved, for instance genetic differences exist as the sheep were outbred and this might affect the immune response to an infection. Furthermore doses of oocysts were made by a dilution process and not by counts, therefore it cannot be guaranteed that each ewe received 200 oocysts. In addition non-specific factors such as stomach contents at the time of ingestion and fluid intake may also have had effects.

Since the IgG -ELISA had not been standardised, it was thought necessary to compare the results from it with another more established test. The IHA test performed at the V.I. Centre in Edinburgh was chosen for several reasons. Firstly the antigen preparations used in both tests are similar, a soluble preparation of RH strain tachyzoites and secondly this test has been used in

previous ovine experimental infections (Blewett et al, 1982; Blewett et al, 1983; Miller et al, 1982; Teale et al, 1982). which facilitates comparisons between published experiments and those recorded in this thesis.

As expected the correlation between the two tests was substantial, although it should be remembered that the IHA test detects both IgM and IgG and therefore it may show a rise in titre sooner than the IgG-ELISA.

CHALLENGE AND LAMBING

Eleven ewes were infected with BD virus either before or during pregnancy and four of these produced congenitally infected lambs. During the period when infection occurred these animals had been kept in the same sheep house as ewes experimentally infected with BD virus. It therefore seems reasonable to conclude that these animals were the source of the infection. The transmission could have occurred by contact with the virus either in the passageway or on fomites brought into the pen by experimental workers. These findings emphasise the need for strict control measures both in the design and execution of experiments involving infectious agents.

All the ewes which were subsequently found to be infected with BD virus had previously been inoculated with 2000 T.gondii oocysts and had already developed an immune response to the parasite. The results indicated that infection with BD virus did not compromise their response to a challenge infection during pregnancy. However it should be noted that live lambs born to ewes infected with BD during pregnancy had low birth weights and it was considered legitimate to remove them when weights were compared between groups.

All the sheep which were inoculated with 2000 oocysts before pregnancy lambled normally and there was no indication that congenital infection with the parasite had occurred. This agrees with other workers who reported that infection before pregnancy does not lead to uterine transmission (Beverley and Watson, 1971; Buxton and Finlayson, 1986).

When animals which had initially received 2000 oocysts were challenged, no pyrexia was recorded, which suggests that on the latter occasion no parasitaemia took place. In addition the level of specific antibody present in the ewes sera was unaltered following challenge. Both these observations have previously been reported following experimental infection of sheep with tissue cysts (Blewett et al, 1982; Buxton and Finlayson, 1986). Hence the data obtained during this study suggests that infection induced by injection of tissue cysts produces the same clinical and serological pattern as the natural mode of infection. The lambs born in this group did not exhibit any signs of Toxoplasma infection, thus it can be concluded that an oral dose of 2000 M1 strain oocysts before pregnancy is sufficient to induce an effective immune response which is then able to neutralise any parasite challenge before it can reach the uterine tissues.

All animals which received 20 or nil oocysts and those which did not react to the 200 oocysts inoculum responded both clinically and serologically to the challenge inoculum. Several abortions occurred in these groups soon after the pyrexia had ended and although the brains of these foetuses were too autolytic for histology, examination of the available placentae did not reveal focal necrosis. It is recognised that fever can induce spontaneous

abortions (Kline, Stein, Susser and Warburton, 1985) and considering both the time scale involved and the absence of characteristic Toxoplasma lesions, it is possible that this was responsible for the fatalities.

Other ewes lost their lambs between 119 and 143 days gestation and examination of brains and placentae revealed the characteristic signs of Toxoplasma induced abortions. Diagnosis was successful in most cases either by histology or serology, however in some instances this was not possible and it is only assumed that lamb loss was due to Toxoplasma infection.

Live lambs born in these three groups were of low birthweight and poor condition and required assistance with feeding during the initial period following birth.

It is apparent that ingestion of 20 Ml strain oocysts and in some instances 200 oocysts is insufficient to protect against challenge in any way. Furthermore not only does primary infection with 10,000 oocysts cause considerable fatalities, in the conceptus, but the live offspring also have significantly lower birthweights than uninfected lambs and are less likely to survive unaided.

The three ewes which had responded serologically after the initial infection continued to have high levels of antibody after the challenge infection. The ewe which initially had no clinical signs developed a pyrexia upon challenge with 10,000 oocysts and then went on to produced two live lambs. Despite the presence of anti-toxoplasma antibody the ewe's offspring were congenitally infected with Toxoplasma as determined by serology. Of the other

two animals, one aborted at 114 days of gestation but since no tissue or serum was obtained it is difficult to say what caused the abortion in this case. The remaining ewe produced two live lambs in which Toxoplasma infection was not detected.

Analysis of the serological data from control animals, revealed that one ewe had detectable IgG against T.gondii, three weeks after the challenge infection had been given, therefore it presumably became infected around two weeks beforehand. Beverley and coworkers (1975) found that two lambs developed anti-toxoplasma antibody after being housed with orally infected animals. Furthermore mice orally infected with T.gondii oocysts can shed approximately 10 per cent of the dose in their faeces within 24 hours of inoculation (Dubey and Frenkel, 1973).

It therefore is feasible that this ewe become infected following the ingestion of oocysts shed by its penmates. However why oocysts should pass through the gastrointestinal system without excysting and then excyst in another animal's intestine is unclear. This ewe transmitted the infection vertically and its lamb was born underweight and with a high level of anti-toxoplasma antibody in its serum.

Although it is possible that this could occur in natural outbreaks it is more likely that infection of large numbers of animals would be due to ingestion of oocysts from infected cat faeces than from contaminated sheep faeces.

CONCLUSIONS

The results described here demonstrateⁿ that 2000 M1 strain oocysts are capable of inducing a pyrexia and seroconversion in all recipient animals. Furthermore this dose is sufficient to induce a protective immune response. Ewes that received 20 or no oocysts before pregnancy were susceptible to challenge and severe lamb loss occurred. Two hundred oocysts appears to be the threshold value for the induction of Toxoplasma infection in sheep, although not all animals that seroconverted were protected from further challenge.

TABLE 4.1 Experimental design for the titration and challenge of
Toxoplasma gondii oocysts in sheep.

56 DAYS PRIOR TO MATING

80 DAYS GESTATION

GROUP	NO. OF EWES	ORAL DOSE OF <u>T. GONDII</u> OOCYSTS	GROUP	NO. OF EWES	ORAL DOSE OF <u>T.GONDII</u> OOCYSTS
1	19	2000	1A	7	NIL
			1B	8	10,000
2	10	200	2	10	10,000
3	11	20	3	10	10,000
			4A	11	10,000
4	19	NIL	4B	5	NIL

TABLE 4.2 Outcome of pregnancies in ewes given Toxoplasma gondii oocysts.

GROUP	NO.OF EWES	NO.OF EWES WITH > 1 LIVE LAMB	TOTAL NO. LAMBS POSSIBLE	LIVE	EARLY ABORT.	LATE ABORT.
1A	7	7 (100)+	10	10 (100)	-	-
1B	8	7 (87.5)	11	10 (91)	-	1 (9)
2	10	4 (40)	16	6 (37.5)	2 (12.5)	7(43.8)
3	10	2 (20)	15	2 (13.3)	7 (46.7)	4(26.7)
4A	11	3 (27.3)	15	4 (26.7)	4 (26.7)	6 (40)
4B	5	5 (100)	7	7 (100)	-	-

+ No. in parenthesis refers to percentage values.

TABLE 4.3 Proportion of live lambs with histological and/or serological evidence of Toxoplasma infection.

GROUP	NO. EWES	NO. PLACENTAE RECOVERED	<u>TOXOPLASMA</u> LESIONS +	NO. PRECOLOSTRAL SERA COLLECTED	ANTI-TOXOPLASMA IgG ANTIBODY
				NO. LAMBS BORN	
1A	7	5	0 (0)*	8/10	0 (0)
1B	7	5	0 (0)	10/10	0 (0)
2	4	3	2 (66)	6/6	5 (83)
3	2	2	2 (100)	2/2	2 (100)
4A	3	2	2 (100)	4/4	4 (100)
4B	5	4	0 (0)	7/7	1 (14.3)

+ As defined by focal necrosis

* No in parenthesis correspond to percentage

TABLE 4.4 Mean weights of live lambs born in each treatment group and these weights adjusted for the effect on twins.

AVERAGE WEIGHTS OF LAMBS		
GROUP	AVERAGE	WEIGHT ADJUSTED FOR TWINS
1A	4.68	4.70
1B	4.54	4.51
2	3.85	3.98
3	3.60	3.21
4A	2.98	3.05
4B	4.54	4.50

TABLE 4.5 Proportion of abortions and stillbirths with histological and serological evidence of Toxoplasma infection.

GROUP	NO. EWES	NO. PLACENTAE RECOVERED	PLACENTAL HISTOLOGY		BRAIN HISTOLOGY		SEROLOGY	
			<u>TOXOPLASMA LESIONS</u>	<u>TOXOPLASMA LESIONS</u>	<u>NO. PRECOLOSTRAL SERA COLLECTED</u>	<u>ANTI-TOXOPLASMA IgG ANTIBODY</u>		
			<u>NO. FOETUSES</u>					
1A	-	-	-	-	-	-	-	
1B	1	1	0 (0)	1	0 (0)	1/1	0 (0)	
2	6	4	3 (75)	5	5 (100)	3/10	2 (66)	
3	8	5	2 (40)	3	3 (100)	2/13	2 (100)	
4A	8	6	5 (83)	6	6 (100)	4/11	1 (25)	
4B	-	-	-	-	-	-	-	

Figure 4.1 Mean rectal temperature response following oral inoculation with Toxoplasma gondii oocysts and standard errors.

—— 2000 oocysts
—— 200 oocysts
—— 20 oocysts
—— nil oocysts

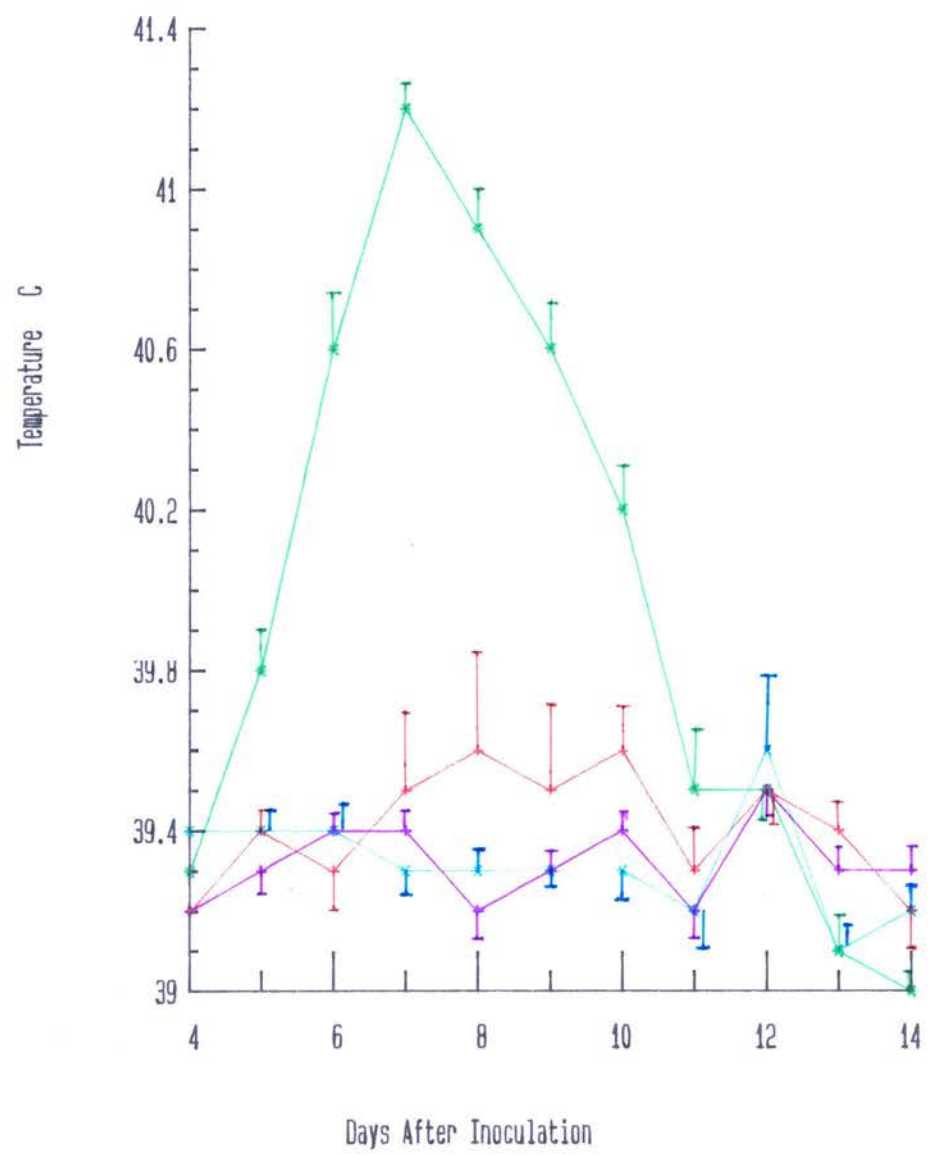


Figure 4.2 Temperature response of sheep
given 200 Toxoplasma gondii
oocysts divided into responders ———
and non responders. ———

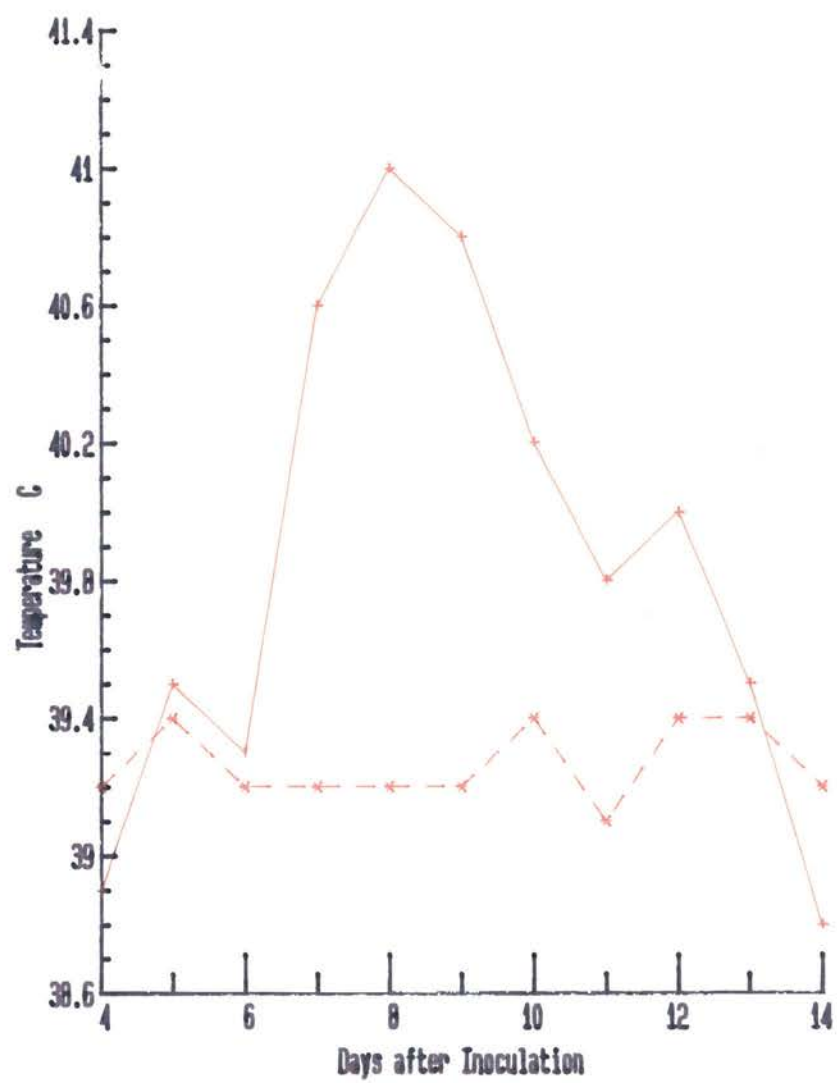
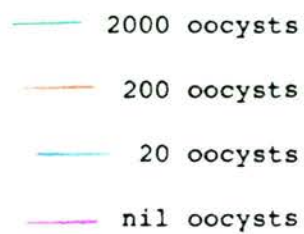


Figure 4.3 IgG antibody against Toxoplasma
(mean of per cent O.D.) detected
in the serum of sheep inoculated
with oocysts. (+ standard errors)



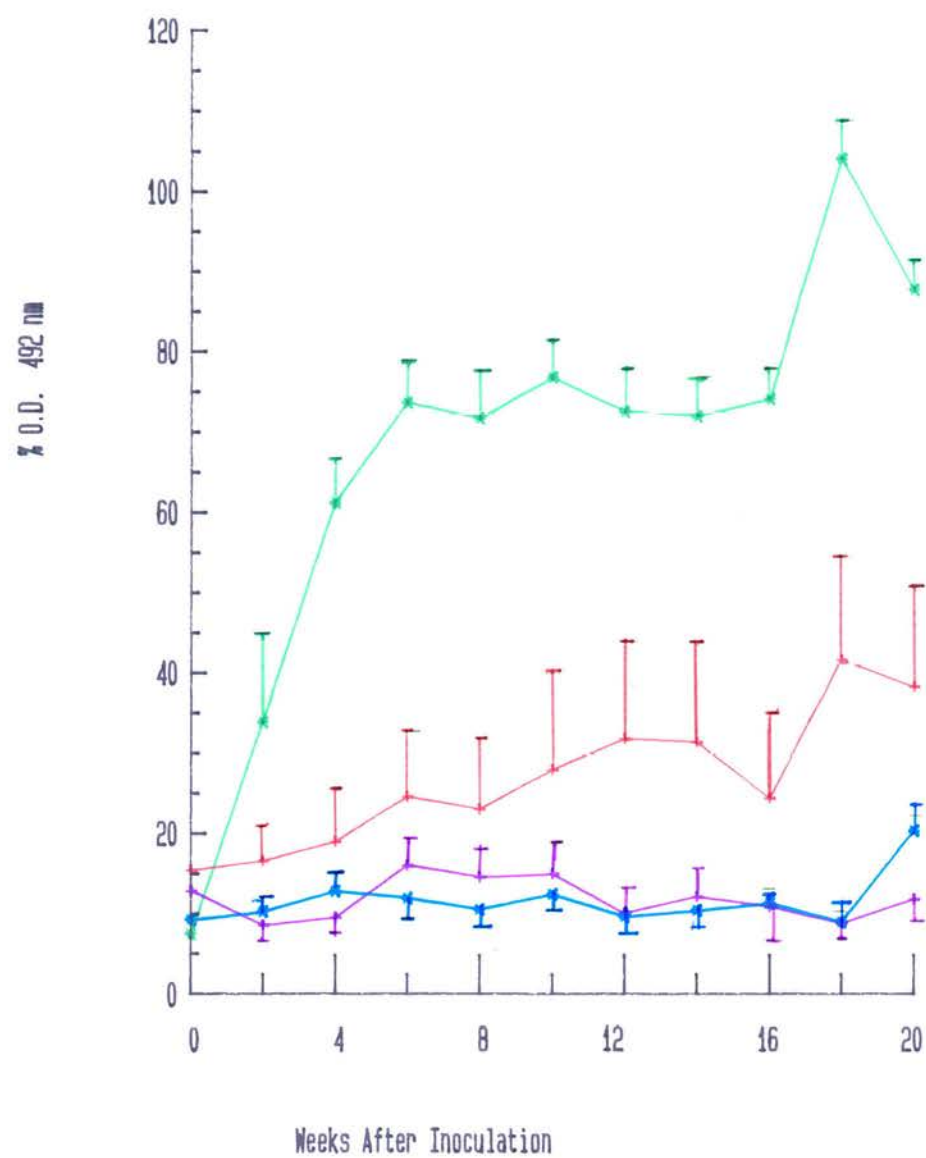


Figure 4.4 IgG antibody response of ewes
given 200 Toxoplasma gondii
oocysts, divided into responders ———
and non responders. ———

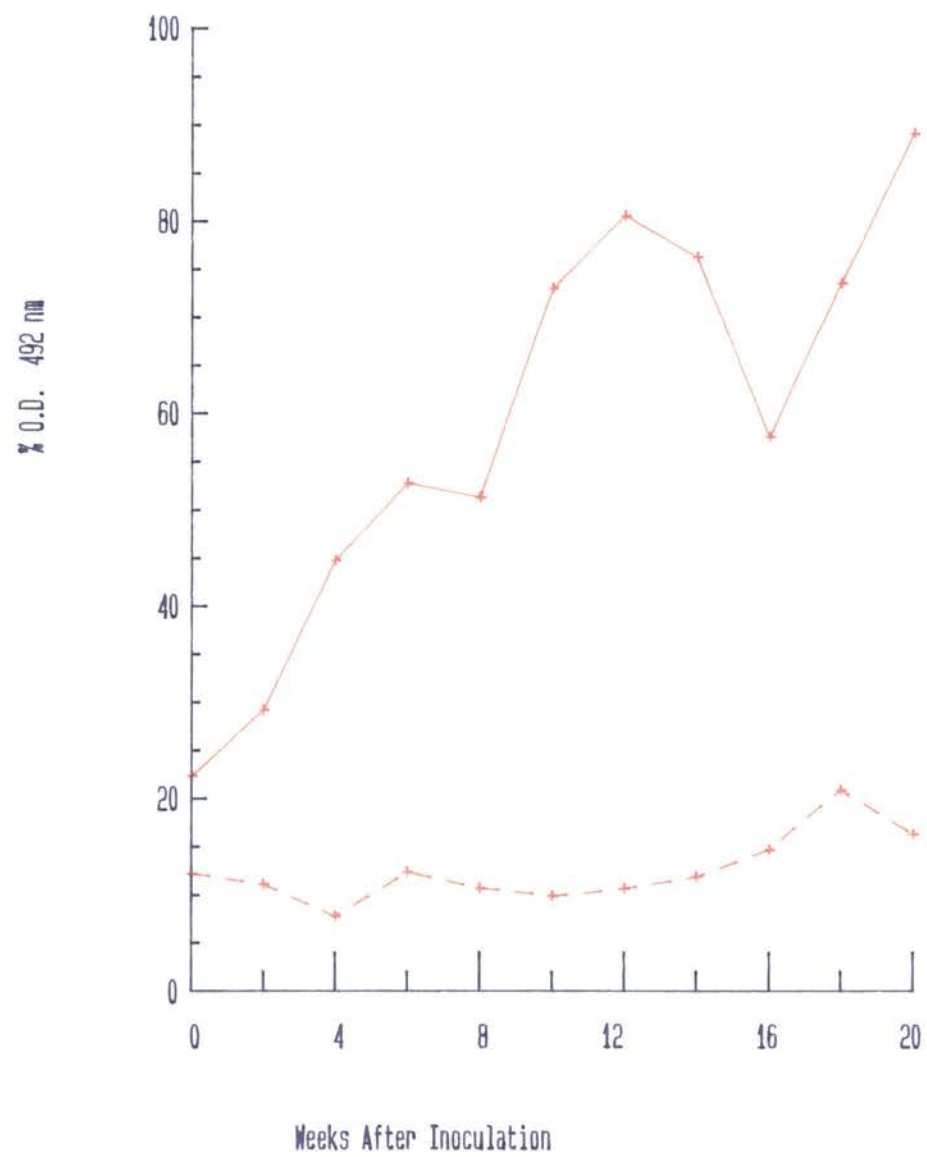


Figure 4.5 Rectal temperatures of ewes challenged with 10,000 Toxoplasma gondii oocysts at mid-gestation (Mean values and standard errors).

— 2000/10,000 oocysts
— 2000/nil oocysts
— 200/10,000 oocysts
— 20/10,000 oocysts
— nil/10,000 oocysts
— nil/nil oocysts

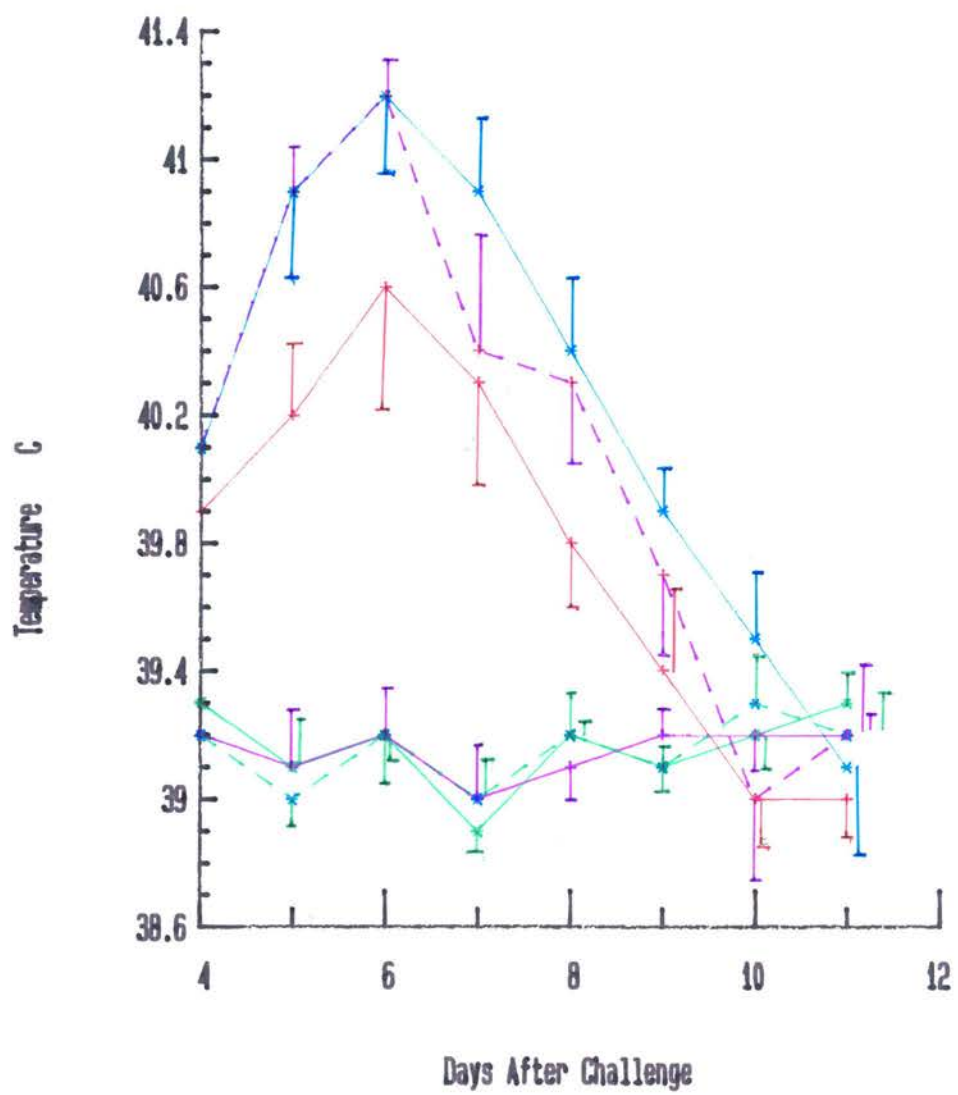


Figure 4.6 Rectal temperatures of ewes
initially given 200 oocysts after
challenge with 10,000
original responders
original non responders

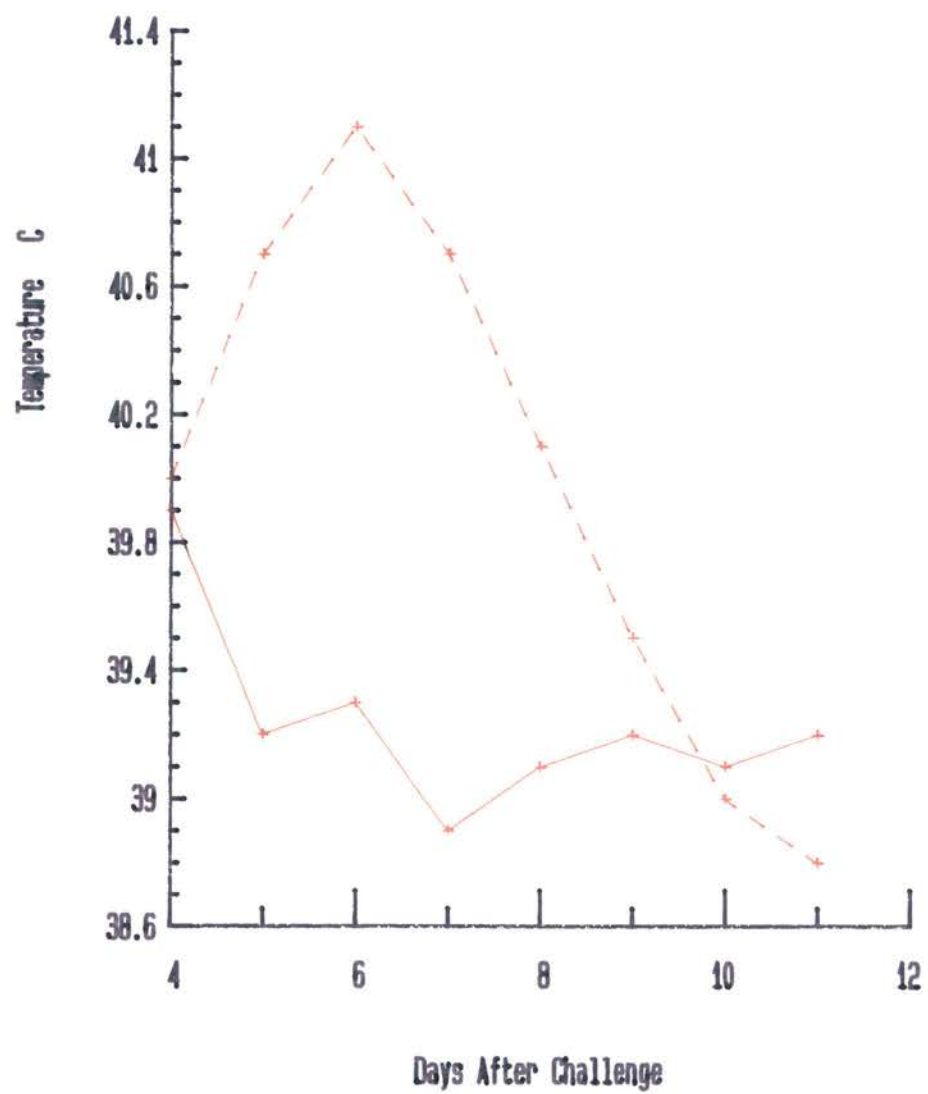


Figure 4.7 Anti-toxoplasma IgG detected in
the sera of ewes challenged with
10,000 Toxoplasma gondii oocysts.
(mean values + standard errors)

---	2000/10,000 oocysts
—	2000/ nil oocysts
---	200/10,000 oocysts
---	20/10,000 oocysts
---	nil/10,000 oocysts
---	nil/nil oocysts

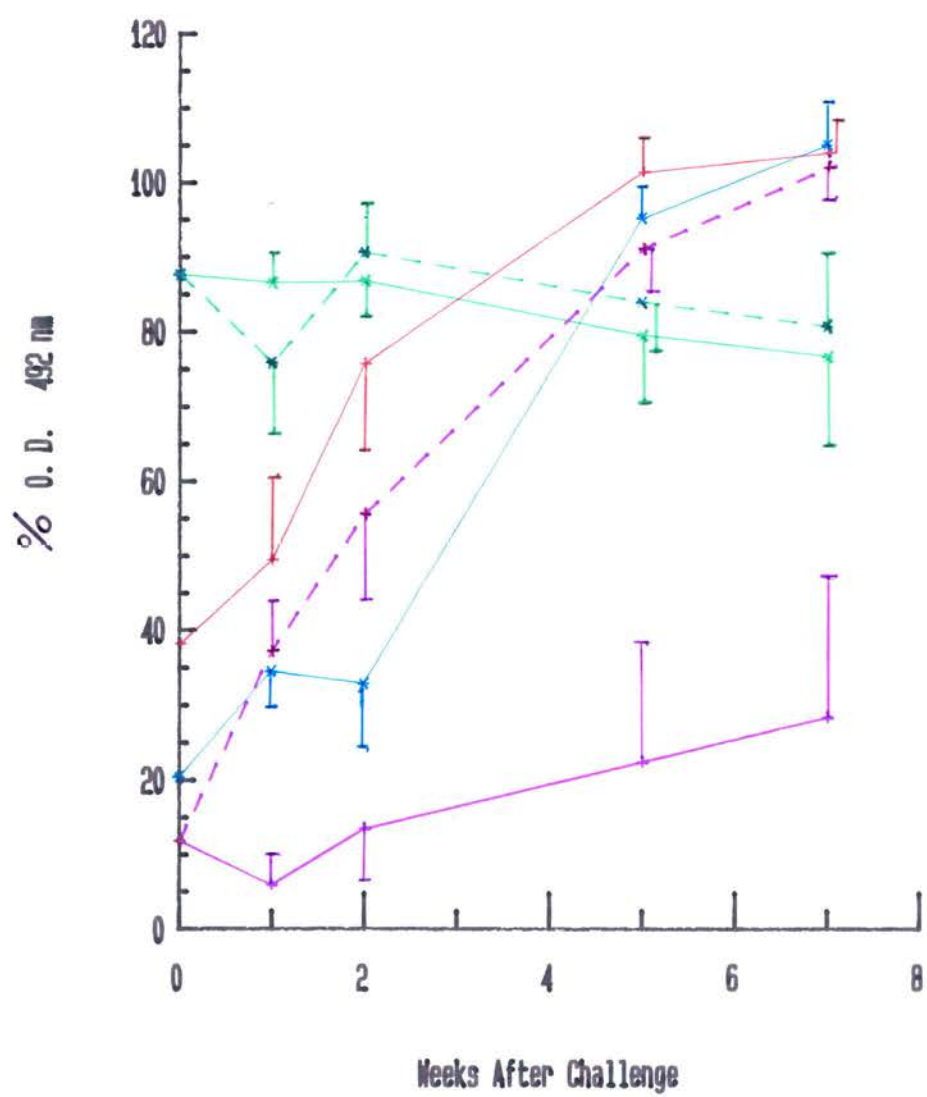


FIGURE 4.8 Placental cotyledons from
 Toxoplasma infected foetus showing
 characteristic white spots
 indicative of focal necrosis.

X 600

FIGURE 4.9 Microscopic view of focal necrosis
 in Toxoplasma infected placental
 cotyledons stained by H & E.

X 600

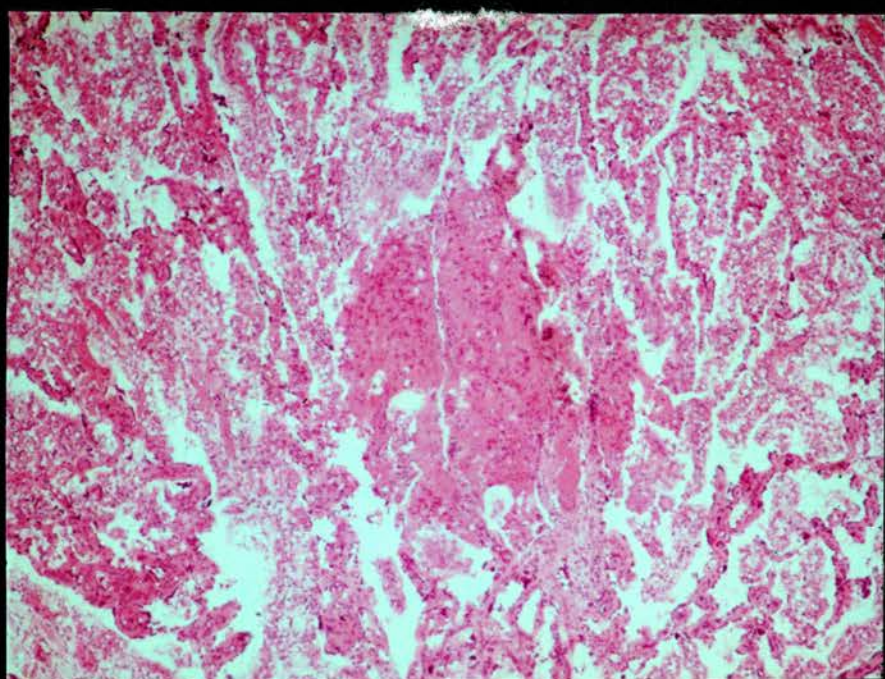
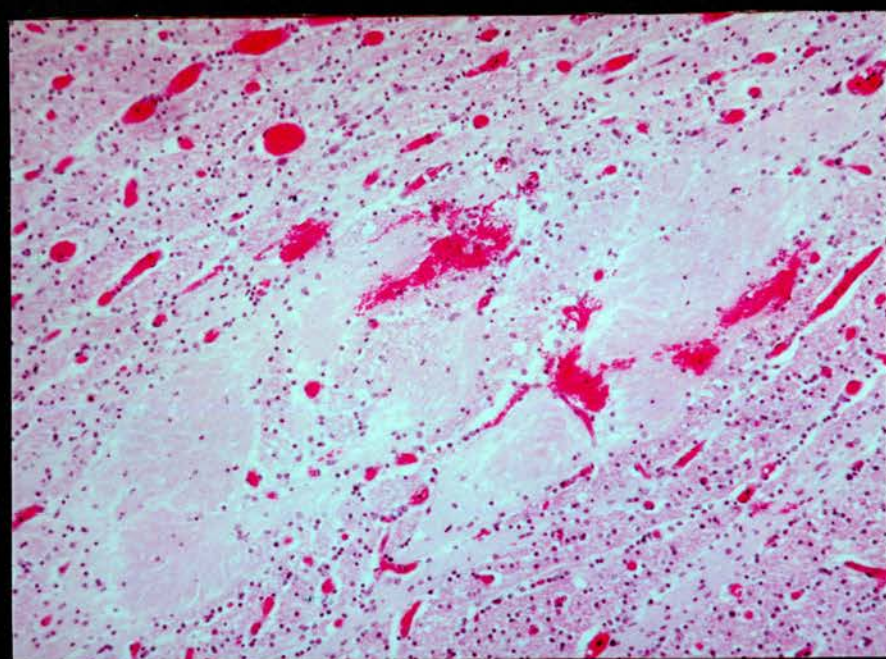
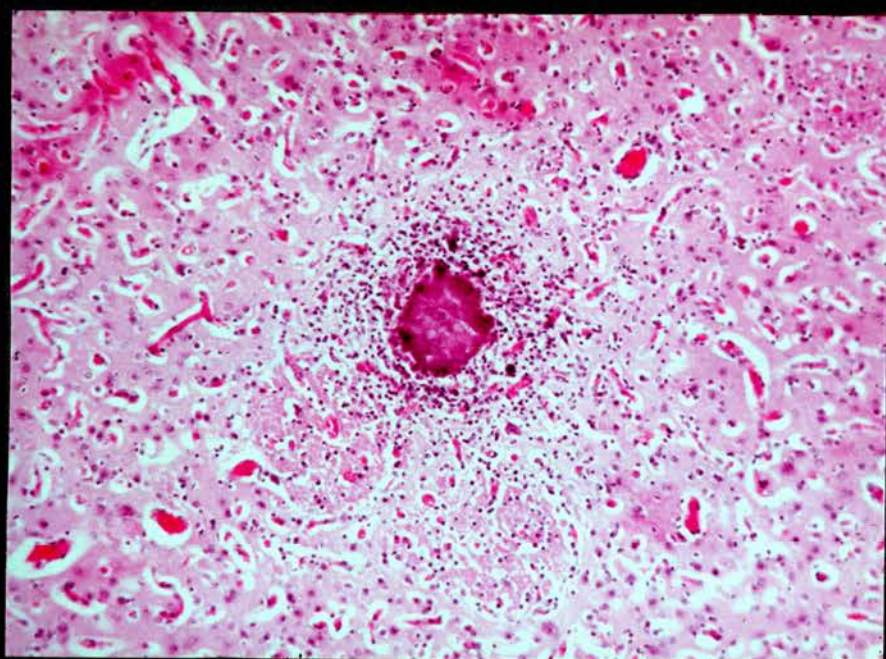


FIGURE 4.10 Focal inflammation in brain of
foetus infected in utero with
Toxoplasma stained by H & E.

X 600

FIGURE 4.11 Leucomalacia in brain of foetus
infected in utero with Toxoplasma.
Section stained by H & E.

X 600



CHAPTER 5 The effect of γ -irradiation on the ability of
Toxoplasma gondii oocysts to induce an immunological
response in mice.

INTRODUCTION

Immunity to Toxoplasma occurs readily in most species after natural infection, however on occasions experimental inoculation of very large numbers of virulent organisms can overcome this protection and result in reinfection (Beverley and Watson, 1971). Attempts to induce immunity in mice with killed organisms have been mostly unsuccessful although the use of adjuvants (Krahenbuhl et al, 1972; Waldeland and Frenkel, 1983) and repeated inoculations (Nakayama, 1969) increases the likelihood of obtaining protection. In addition the use of experimental animals which have a natural immunity to Toxoplasma infection, such as guinea pigs, also aids the success rate (Foster and McCulloch, 1968). Inoculation with killed Toxoplasma can induce an antibody response in rabbits, guinea pigs and mice (Huldt, 1966; Foster and McCulloch, 1968; Krahenbuhl et al, 1972) however this does not relate to the ability of the animals to resist challenge with virulent organisms.

Evidence to date therefore indicates that a live infection must occur before effective long lasting immunity can be established, however this leads to a chronic infection in most species which may become reactivated later if the host's immunity is suppressed. Since tissue cysts are infectious if ingested, a source of infection for other animals is produced. Encouraging results were reported in 1983 (Waldeland and Frenkel) on the use of a mutant "non-persisting" strain of Toxoplasma for mouse immunisation which removed the two drawbacks stated above, however no subsequent work has been published.

Another method which has previously been used to alter the virulence of both helminth and protozoan parasites is radiation.

Jarrett and his coworkers attenuated Dictyocaulus viviparus by X-irradiation and found that oral inoculation induced a substantial degree of protection against subsequent live challenge in both mice and cattle (Jarrett, Jennings, Martin, McIntyre, Mulligan, Sharp and Urquart^h, 1958; Jarrett, Jennings, McIntyre, Mulligan and Sharp, 1961). Furthermore inoculation of γ -irradiated red blood cells infected with Babesia rodhaini induced immunity to challenge infection in both mice and rats (Philips, 1971). Another protozoan parasite, Eimeria tenella, can induce severe pathological effects in chickens, however Hein (1963) found that prior administration of X-irradiated oocysts in drinking water evoked substantial protection against subsequent challenge with live oocysts, as determined both by the absence of clinical symptoms and the low numbers of oocysts produced.

More recently Toxoplasma tachyzoites have been irradiated by a γ source (Seah and Hucal, 1975; Chhandra et al, 1979) and the same workers demonstrated that up to 100 per cent of the mice immunised could resist a virulent challenge 30 days later. These results were obtained after only one inoculation and are therefore more encouraging than the results previously described with killed preparations.

No previous studies have reported the effects of γ -irradiation on Toxoplasma oocysts and their ability to induce protective immunity, therefore it was considered to merit investigation. Before γ -irradiated oocysts could be tested in sheep, it was thought to be necessary to establish in mice the effects of inoculation of oocysts treated with various doses of irradiation.

Therefore the aims of this study were to establish if i.p. inoculation of T.gondii oocysts treated with various doses of γ -irradiation would protect mice against a subsequent lethal challenge and if so, how soon after the initial inoculation this immunity could be evoked. Additionally it was my intention to relate these findings to the presence of both serum anti-toxoplasma antibody and tissue cysts in mouse brain.

EXPERIMENTAL PROCEDURE

Experiment 1

Toxoplasma gondii oocysts were irradiated with either 5, 10, 20 or 40 krad from a cobalt source (Chapter 2). Mice in groups of 10 were inoculated with 500 irradiated oocysts, whilst a further five mice received 500 untreated oocysts and another group (five mice) were not injected (Table 5.1). A challenge inoculum of RH strain tachyzoites was prepared as described in Chapter 2, and 28 days after the initial inoculation each mouse was given 2×10^5 organisms.

Experiments 2 and 3

A further two experiments were carried out which had a similar design to experiment 1. However only two doses of irradiation were used and the groups of mice were challenged either nine or 19 days after the original inoculation (Table 5.2).

Experiment 4

Four doubling dilutions were prepared from a stock solution which contained 2.5×10^3 oocysts / ml. Groups of 16 mice were injected i.p. with each dose, whilst a further 16 mice were left

untreated. After 28 days all the mice received the RH strain challenge inoculum (Table 5.3).

All the animals in these experiments were monitored daily for clinical signs and mortality.

Experiment 5

Untreated and irradiated oocysts were injected into groups of 10 mice, whilst a further group (10 mice) was left uninoculated (Table 5.4). Nine, 19 or 28 days after inoculation, three or four mice from each group were killed, their blood collected and their brains removed. Sera obtained after the centrifugation of clotted blood was tested with the mouse IgG-ELISA. Giemsa stained smears of brain homogenate were prepared as previously described (Chapters 2 and 3) and examined for the presence of Toxoplasma tissue cysts.

RESULTS

Experiment 1: mouse mortality after day 28 challenge

By day 7 after challenge there were no survivors in the groups which received oocysts irradiated with either 20 or 40 krad before challenge (groups 4 and 5 respectively), or those given only the RH strain tachyzoites (group 6) (Fig 5.1). Those inoculated with oocysts, irradiated with 10 krad (group 3) survived for a longer time than groups 4 and 5 ($P < 0.01$), but by 12 days after challenge they were all dead. In the groups that received either oocysts treated with the minimum irradiation dose or those given unaltered oocysts 40 (group 1) and 30 (group 2) per cent of the mice survived the challenge infection. This mortality rate was statistically lower than the other groups ($P < 0.05$) and furthermore survival of

mice in groups 1 and 2 were more prolonged than the other groups (groups 1 or 2 vs groups 4 or 5, $P < 0.01$ groups 1, 2 or 3 vs group 6 $P < 0.05$).

Experiment 2: mouse mortality after day 19 challenge

Death occurred in all mice injected with only RH strain tachyzoites by seven days after inoculation and those mice that had previously received 10 krad irradiated oocysts did not survive for more than nine days (Fig 5.2). In group 2 (5 krad irradiated oocysts) all the animals were dead by day 14 after challenge while in group 1 (untreated oocysts) there was a 30 per cent survival rate. The length of survival time was significantly longer in groups 1,2 and 3 than in group 4 (group 1 vs group 4, $P < 0.01$; groups 2 or 3 vs group 4, $P < 0.05$).

Experiment 3: mouse mortality after day 9 challenge

Mice only given the challenge inoculum were all dead eight days later whilst in the groups inoculated with either 10 krad irradiated oocysts or untreated oocysts some mice survived until 12 days after challenge (Fig. 5.3). Ten per cent of mice that received 5 krad irradiated oocysts survived the challenge inoculum and the length of survival was significantly prolonged in both this group and in group 1 compared to group 4 ($P < 0.01$).

Experiment 4: mouse mortality after challenge at 28 days

In all the groups inoculated with oocysts a proportion of animals survived the challenge infection (Fig 5.4) whilst all the mice which received only the RH strain tachyzoites were dead seven days later, a finding which is statistically significant (groups 1,3 or 4 vs group 5, $P < 0.01$; group 2 vs group 5, $P < 0.05$).

Experiment 5:

Detection of anti-toxoplasma antibody

No anti-toxoplasma IgG was detected in the sera of animals given 20 or 40 krad irradiated oocysts at any time subsequent to inoculation (Fig. 5.6). Sera obtained 19 and 28 days after injection from groups that received untreated or 5 or 10 krad irradiated oocysts were positive in the mouse IgG-ELISA.

Detection of tissue cysts in mouse brain

No Toxoplasma tissue cysts were detected in the brains of the control mice or from the groups that received oocysts irradiated with 10, 20 or 40 krads (Fig. 5.5). Brains collected 19 and 28 days after challenge from mice that received untreated oocysts all contained tissue cysts whilst in the group given 5 krad irradiated oocysts only one mouse killed on day 19 or day 28 after infection had tissue cysts.

DISCUSSION

The results from the challenge experiments showed that all mice given only 2×10^5 RH strain tachyzoites i.p. were dead seven or eight days after inoculation and prior inoculation with M1 strain oocysts that had been irradiated with either 20 or 40 krad did not alter this outcome. However mice that received 10 krad irradiated oocysts survived for a significantly longer time after challenge than animals in these other three groups. Thirty to 40 per cent of mice that received 5 krad irradiated or untreated oocysts survived the challenge at day 28 and furthermore even when challenged nine days after inoculation these groups of mice survived for a significantly longer time (up to 6 days longer) than mice that

received either oocysts irradiated with 20 or 40 krad or those given only the challenge.

The RH strain was chosen as the challenge inoculum because not only does it give a clear clinical outcome but also it is routinely passaged at MRI and the timing of the clinical response to i.p. inoculation with 2×10^5 tachyzoites is known. Other workers have used less virulent strains (Krahenbuhl et al, 1972) or smaller numbers of organisms (Nakayama, 1969; Chhandra et al, 1979) as their challenge inoculum and this makes comparison of survival rates more difficult. However Nakayama demonstrated that between 30 and 50 per cent of mice immunised five times with a killed preparation of tachyzoites survived a challenge of 3×10^3 RH strain tachyzoites. Whilst 30 per cent of mice that received a similar immunisation programme remained alive after a challenge with the C56 strain which has a LD 70 at 20 days (Krahenbuhl et al, 1972). Seah and Hucal (1975) irradiated RH strain tachyzoites with 10 krad and found that mice injected i.p. with the tachyzoites were protected against subsequent challenge with 10^4 organisms of the same strain four weeks later. If however higher doses of radiation were used (15 or 20 krad) 80 per cent of the immunised mice survived the challenge. A further study which used the same three irradiation doses demonstrated that prior immunisation with 10 krad irradiated tachyzoites enabled 40 per cent of mice to survive a challenge with 10^5 RH strain tachyzoites while there were no survivors in the groups which received tachyzoites treated with higher doses of irradiation (Chhandra et al, 1979). When the size of the challenge inoculum was reduced by one log, all the groups showed a degree of survival ranging from 60 - 100 per cent. Since

in this study 2×10^5 organisms were used as the challenge then the number of mice surviving after challenge at 28 days is encouraging.

Chhandra and his coworkers (1979) also reported that when mice were challenged two to three weeks after the initial inoculation the organisms which received the higher doses of irradiation induced more protection. Neither the first report (Seah and Hucal, 1975) nor any of my data support this finding. No explanation was offered and since no statistical analysis was reported it is difficult to ascertain the significance of this finding.

In the present study it is not known whether the irradiation altered the virulence of all the oocysts or merely killed a proportion of them. It was thought that this question might at least be partially resolved if mice were inoculated with decreasing doses of viable oocysts to see if a titration of protection was evident after challenge (Expt. 4). However it was found that the four doses of oocysts used, all induced between 40 and 50 per cent survival after challenge, whereas mice that received the challenge inoculum alone all died. Also there was no significant difference in the survival rates between the different groups. Thus a dose response effect is not apparent, although it is possible that doses of oocysts given were not titrated far enough, since in the previously reported titration experiments (Chapter 3) as few as 31 oocysts could induce active infection.

Since no clinical symptoms were manifest after inoculation with irradiated oocysts it was difficult to assess whether a patent infection was taking place. In an attempt to overcome this, groups of mice injected with irradiated oocysts were killed at the same

time intervals as those used for challenge and were examined for specific antibody and tissue cysts. The data indicated that oocysts irradiated with 20 or 40 krad neither induced an antibody response nor caused an infection. However it appears that oocysts irradiated with 5 krad are able to induce seroconversion and also multiply since tissue cysts were found in the brain of a proportion of these animals. Although mice given 10 krad irradiated oocysts developed specific antibody, no signs of chronic infection were found at death. However due to the small numbers of mice used, it is difficult to rule out the possibility that very mild infections may have occurred.

It therefore appears that T.gondii oocysts irradiated with either 5 or 10 krad are able to stimulate a degree of protective immunity against lethal challenge, however the lower dose of irradiation does not appear to alter the oocysts sufficiently to prevent the onset of a chronic infection.

CONCLUSIONS

One i.p. injection of mice with Toxoplasma irradiated oocysts (5 krad, 10 krad) induces a significant degree of protection against a virulent challenge four weeks later whereas oocysts treated with higher doses of irradiation were unable to do so. It is unclear whether irradiation attenuates the oocysts or whether a proportion are killed, however mice injected with 5 krad irradiated oocysts developed a chronic infection. This signifies that organisms still capable of proliferation persist after this dose of irradiation and therefore retain their infectivity.

TABLE 5.1 Design of Experiment 1: Inoculation of mice with Toxoplasma gondii irradiated oocysts and subsequent challenge with RH strain tachyzoites 28 days after initial inoculation.

GROUP	NO. OF MICE	DOSE OF OOCYSTS (IRRADIATION KRAD) DAY 0	CHALLENGE WITH RH TACHYZOITES DAY 28
1	5	500 (NIL)	2×10^5
2	10	500 (5)	2×10^5
3	10	500 (10)	2×10^5
4	10	500 (20)	2×10^5
5	10	500 (40)	2×10^5
6	5	NIL (-)	2×10^5

TABLE 5.2 Design of Experiments 2 and 3: Inoculation of mice with Toxoplasma gondii irradiated oocysts and subsequent challenge with RH strain tachyzoites either nine or 19 days after the initial inoculation.

GROUP	NO OF MICE	DOSE OF OOCYSTS + (IRRADIATION KRAD)	CHALLENGE *
1	10	500 (-)	2×10^5
2	10	500 (5)	2×10^5
3	10	500 (10)	2×10^5
4	10	NIL (-)	2×10^5

+ Day 0

* Day 9 or 19

TABLE 5.3 Design of Experiment 4: Titration of Toxoplasma gondii oocysts in mice and subsequent challenge with RH strain tachyzoites 28 days after infection.

GROUP	NO. OF MICE	DOSE OF + OOCYSTS	CHALLENGE *
1	16	500	2x10 ⁵
2	16	250	2x10 ⁵
3	16	125	2x10 ⁵
4	16	62	2x10 ⁵
5	16	NIL	2x10 ⁵

+ Day 0







* Day 28

TABLE 5.4 Design of Experiment 5: Serial kill of mice inoculated with irradiated Toxoplasma gondii oocysts.

GROUP	NO. OF MICE	DOSE OF OOCYSTS (IRRADIATION KRAD) DAY 0	NO. MICE KILLED DAY 9	NO. MICE KILLED DAY 19	NO. MICE KILLED DAY 28
1	10	500 (-)	3	3	4
2	10	500 (5)	3	3	4
3	10	500 (10)	3	3	4
4	10	500 (20)	3	3	4
5	10	500 (40)	3	3	4
6	10	NIL (-)	3	3	4

FIGURE 5.1 Per cent mortality of mice injected
with irradiated oocysts and
subsequently challenged with RH
strain tachyzoites 28 days later.

Doses were:-

-  500 untreated oocysts
-  500 5 krad irradiated oocysts
-  500 10 krad irradiated oocysts
-  500 20 krad irradiated oocysts
-  500 40 krad irradiated oocysts
-  challenge only

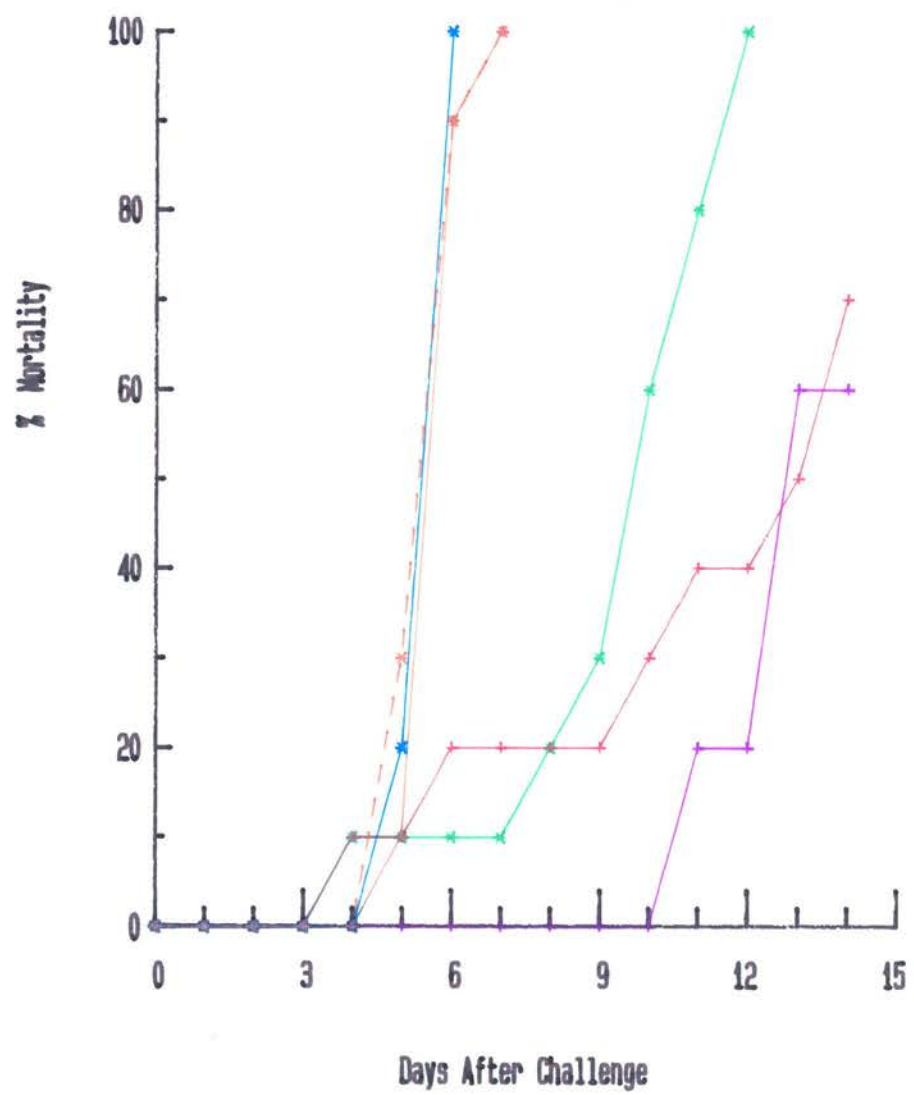


FIGURE 5.2 Per cent mortality of mice infected with irradiated oocysts and subsequently challenged with RH strain tachyzoites 19 days later.

- 500 untreated oocysts
- 500 5 krad irradiated oocysts
- 500 10 krad irradiated oocysts
- challenge only

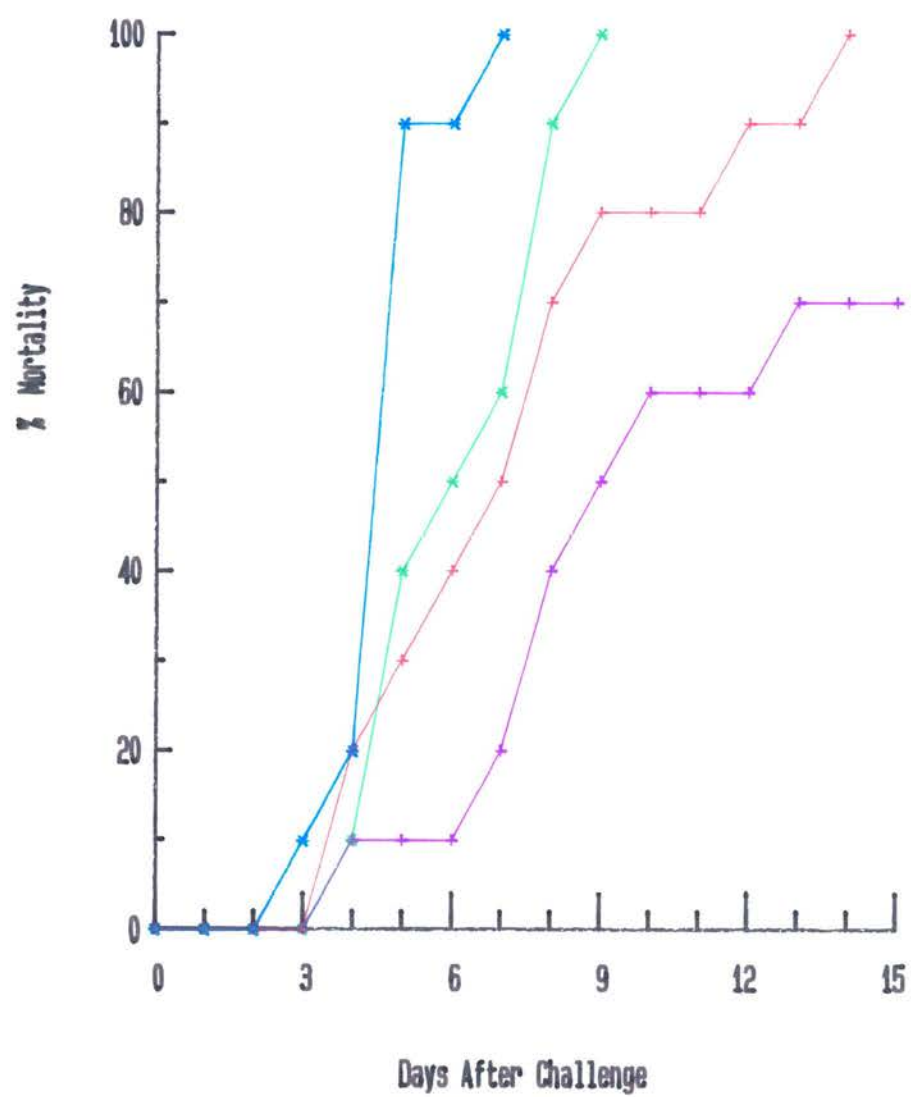


FIGURE 5.3 Per cent mortality of mice injected
with irradiated oocysts and
subsequently challenged with RH
strain tachyzoites 9 days later.
Treatments were

- 500 untreated oocysts
- 500 5 krad irradiated oocysts
- 500 10 krad irradiated oocysts
- Challenge only

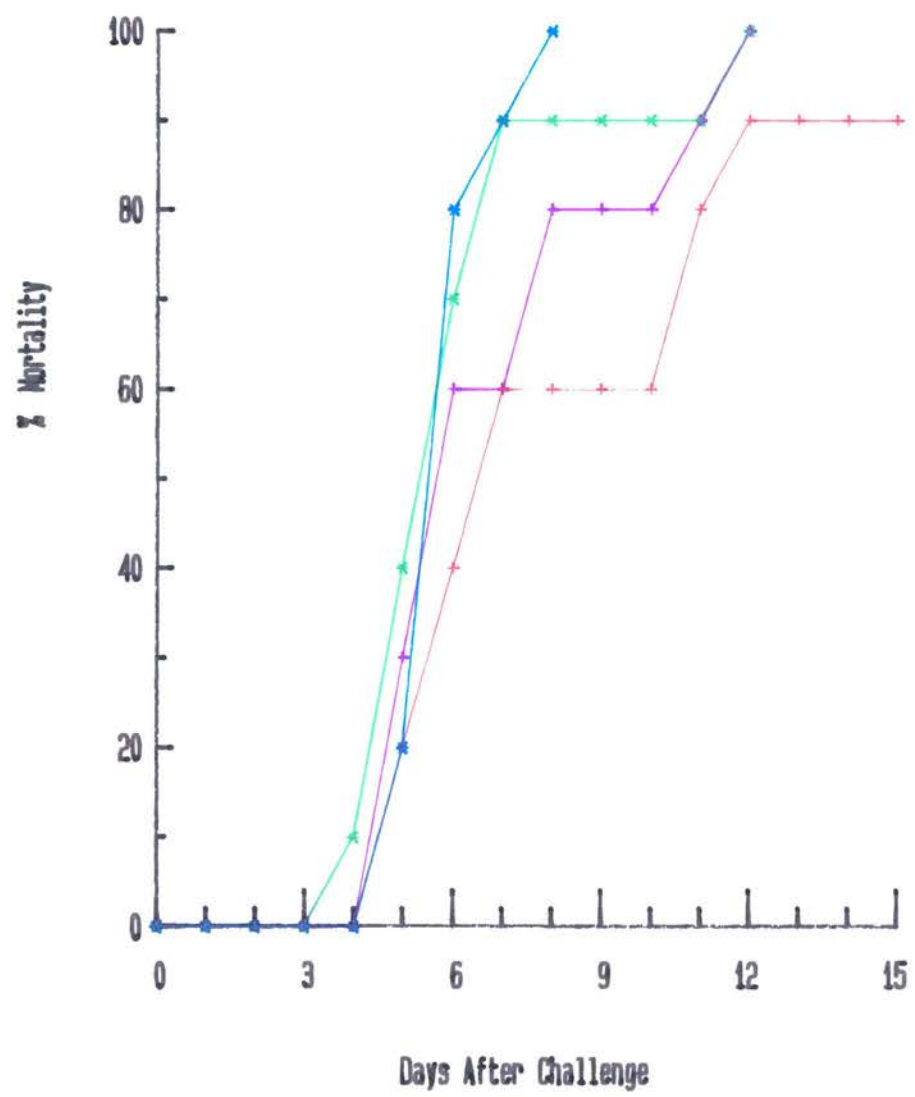







FIGURE 5.4 Per cent mortality of mice injected with doubling dilutions of Toxoplasma gondii oocysts and subsequently challenged after 28 days with RH strain tachyzoites. Doses of oocysts were:-

	500 oocysts
	250 oocysts
	125 oocysts
	62 oocysts
	control

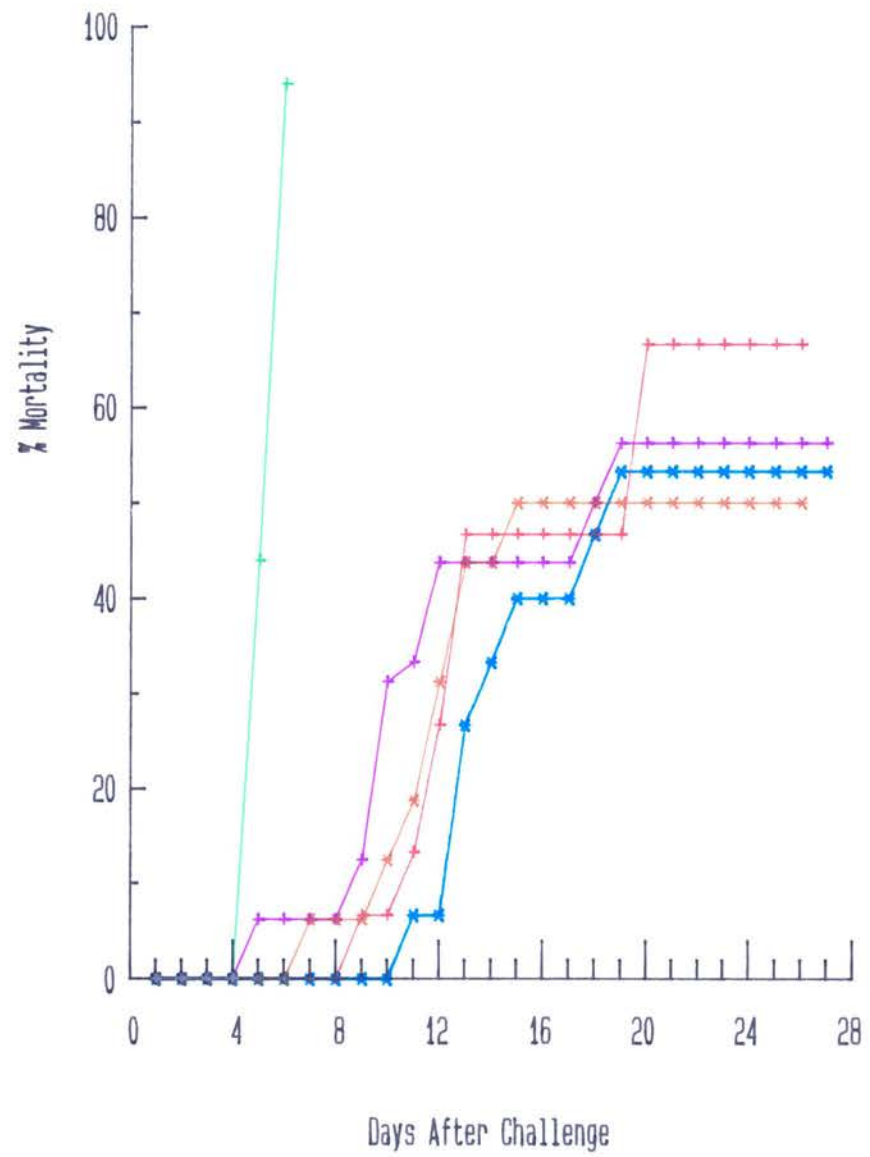


FIGURE 5.5 Mean number of tissue cysts detected
in mice inoculated with irradiated
oocysts by examination of Giemsa
stained brain smears.

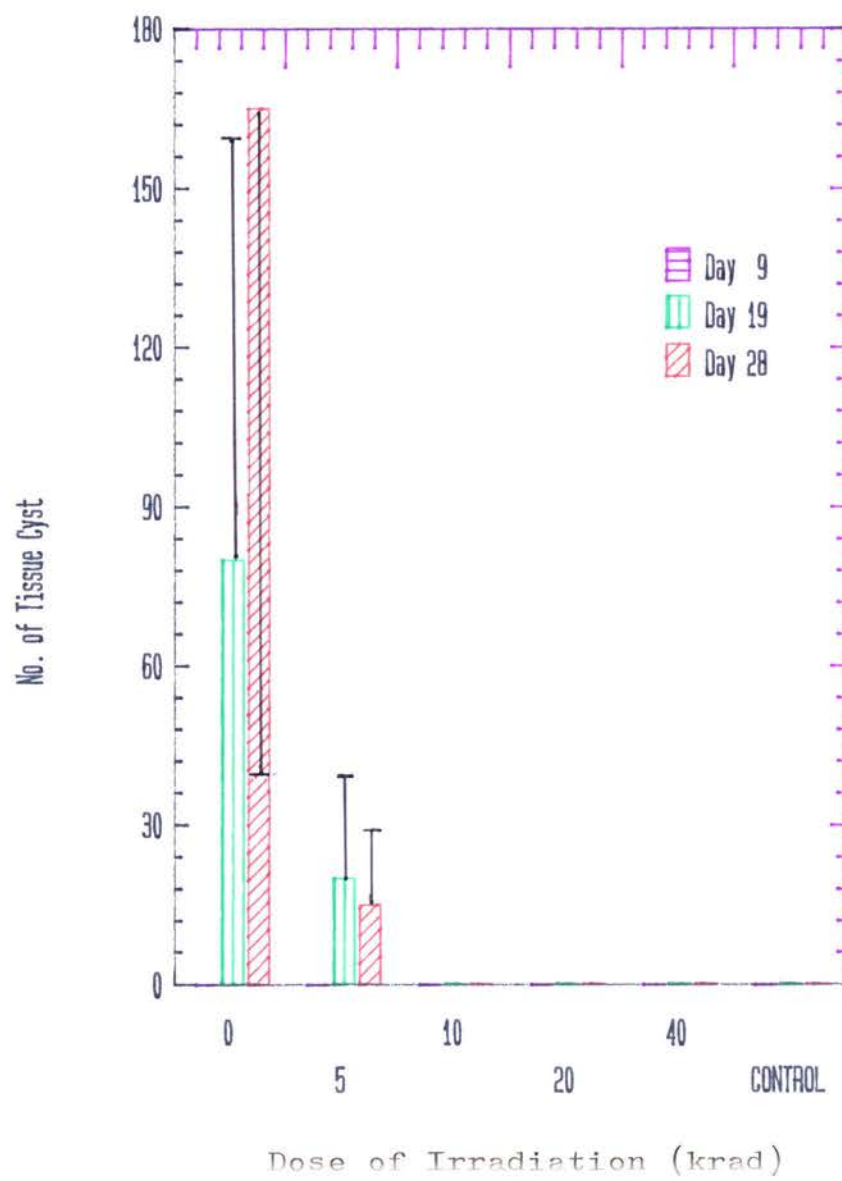
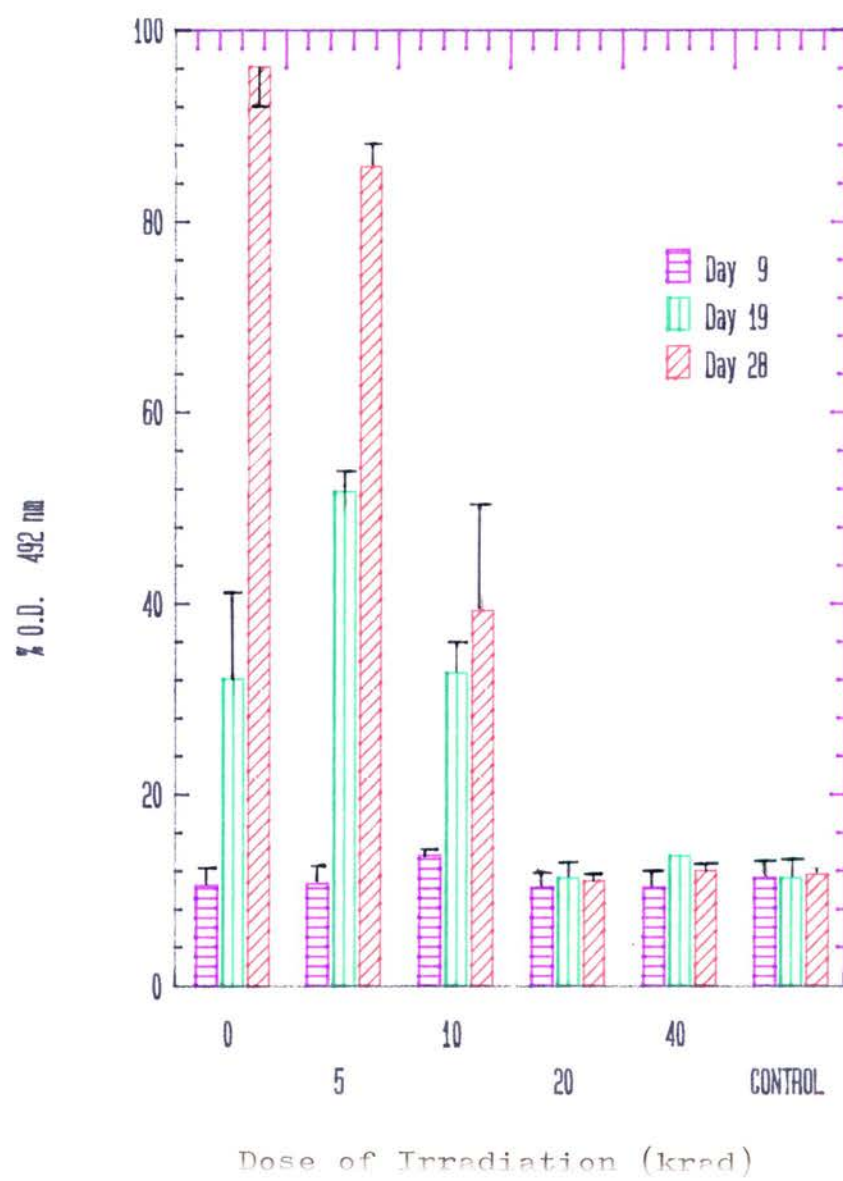


FIGURE 5.6 IgG antibody against Toxoplasma
detected in sera of mice inoculated
with irradiated oocysts (per cent
O.D.).



CHAPTER 6 The antigenicity and pathogenicity of γ -irradiated
Toxoplasma gondii oocysts for sheep.

INTRODUCTION

Toxoplasma infection normally causes only minor illness in sheep and these animals are protected from subsequent challenge (Jacob, 1961; Beverley and Watson, 1971; Blewett et al, 1982; Buxton and Finlayson, 1986). Thus while inoculation with live unaltered organisms outwith pregnancy would cause only minor discomfort to the animal it would prevent the possibility of infection during pregnancy leading to abortion and stillbirths. However this procedure would be unacceptable since it would create a vast potential source of infection for Man and other animals. There have been limited studies on immunoprophylactic control of Toxoplasma infection in sheep and Beverley and coworkers (1971) demonstrated that a killed preparation of Toxoplasma tachyzoites failed to prevent a challenge inoculum of tissue cysts, given during mid-pregnancy, from infecting both placenta and foetus. Since γ -irradiated oocysts produced a significant degree of protection against lethal challenge in mice (Chapter 5) it was decided to carry out further experiments in sheep.

In Chapter 5 only oocysts treated with 5 or 10 krad of irradiation produced significant protection and therefore these doses were used in this study. The aims were two-fold: a) to determine in non-pregnant sheep if one s.c. inoculation with irradiated oocysts will induce protection against subsequent challenge with untreated oocysts and b) to establish if oocysts, irradiated with 5 krad, retain their infectivity and pathogenicity for the ovine foetus.

EXPERIMENTAL PROCEDURE

Experiment 1

Twelve four month old Suffolk x Scottish Blackface female or castrated male lambs were employed in this study. They were born to ewes experimentally infected with Toxoplasma before pregnancy (see chapter 4). Ten of these had no detectable anti-toxoplasma antibody, whilst the other two had antibody to T. gondii when tested with the IgG ELISA, presumably due to maternally transferred antibody.

Animals (two per group) were inoculated with either 2000, 200 or 20 T.gondii oocysts or with 2000 irradiated oocysts (5 or 10 krad) s.c. whilst the two animals with detectable antibody served as controls (Table 6.1). Eight weeks after the initial inoculation all the animals were challenged s.c. with 2000 oocysts.

Rectal temperatures were recorded daily for 22 days after the initial injection and for 13 days after the challenge, whilst blood samples were obtained every four days for the first 12 days after inoculation and subsequently at weekly intervals. Serum was obtained from clotted blood after centrifugation and was tested with the IgG-ELISA

Experiment 2

Three Scottish Blackface ewes were inoculated at 80 days gestation with 2000 irradiated T.gondii oocysts (5 krad) (Table 6.2). Rectal temperatures were recorded daily for 18 days after inoculation and blood samples were collected weekly, until lambing. Sera were obtained from clotted blood and tested with the IgG-ELISA.

Ewes were monitored closely during the lambing period and the placentae from all ewes and brains from all stillborn lambs collected for histopathology. In addition a blood sample was collected from live lambs and the serum from this separated and stored at -20°C until examined for anti-toxoplasma antibody with the IgG-ELISA.

RESULTS

Experiment 1: s.c.inoculation with untreated and irradiated oocysts

Primary infection

Temperature Response

All animals inoculated with T.gondii oocysts developed a pyrexia although in the groups which received 20 oocysts the fever was slower in onset. The peak of the response in animals that received 2000 irradiated oocysts occurred after the peak of the group which received 2000 oocysts. No fever was detected in the two control animals (Fig. 6.1).

Serology

Specific anti-toxoplasma IgG antibody was detected in all animals inoculated with T.gondii oocysts and there was no apparent difference in the time of onset (Fig. 6.2). The amount of detectable anti-toxoplasma IgG antibody decreased in the control animals over this period of time which further suggests that it was maternal antibody.

Challenge Infection

Temperature response

All of the animals previously injected with T.gondii oocysts

remained afebrile after the injection of the challenge inoculum and these results are pooled in figure 6.3. The two control lambs displayed a characteristic temperature response following infection.

Serology

Detectable anti-toxoplasma IgG remained high in the inoculated animals after challenge. The control animals developed specific IgG antibody after infection with 2000 oocysts and this was still rising when the final blood samples were collected (Fig 6.4).

Experiment 2: s.c.inoculation of irradiated oocysts in pregnant ewes

Temperature Response

A temperature response was detected in two out of the three sheep beginning 11 and 12 days after inoculation and lasting for three days (Fig. 6.5).

Serology

Anti-toxoplasma IgG antibody was first detected two weeks after inoculation and this continued to rise until the final sample was taken (Fig. 6.6).

Lambing

Live Lambs

One ewe gave birth to a live lamb which weighed 4.5 kg at birth. Examination of the placental cotyledons revealed focal necrosis which was characteristic of Toxoplasma infection (See Chapter 4). Only post colostrum serum was obtained from this lamb and as expected it gave a positive result when tested for the presence of anti-toxoplasma antibody (Table 6.3).

Dead Lambs

The other two ewes produced three stillborn lambs at 133 and 141 days of gestation. Focal necrosis was present in the placental cotyledons from all the animals and examination of the brains revealed leucomalacia and focal inflammation characteristic of Toxoplasma infection (Buxton et al, 1982) (Table 6.3).

DISCUSSION

In experiment one to permit comparison between the responses of animals given untreated and irradiated oocysts, groups of sheep were injected with several doses of untreated oocysts. The three doses chosen, 2000, 200 and 20 oocysts, were those previously given orally to animals in the titration experiment (Chapter 4).

As few as 20 oocysts injected s.c. caused both pyrexia and seroconversion, whereas when oocysts were given orally at least 200 were required. This is the first report of parenteral administration of T.gondii oocysts in sheep and it demonstrates that as with mice oocysts can excyst in areas other than the intestines (Dubey and Frenkel, 1973). Whether this is due to more efficient excystation or simply a dilution effect in the rumen is not known. All animals given irradiated oocysts developed a febrile response and specific antibody. The onset and duration of the pyrexia seems to be dependent on the dose of oocysts inoculated, a finding previously recorded for tissue cyst infection (Blewett et al, 1982). The temperature response of animals inoculated with 200 oocysts or 5 krad irradiated oocysts was similar. No difference was apparent in the detection of anti-toxoplasma antibody in the groups signifying that even the smallest inoculum was sufficient to induce seroconversion.

A temperature response after infection is indicative of a parasitaemia (Hartley, 1964; Reid et al, 1982) which suggests that the irradiated oocysts are still capable of multiplication, therefore, the possibility exists that immunity to challenge is achieved by a live infection. Previous work with Eimeria bovis has demonstrated that irradiated oocysts have to complete their lifecycle, thereby producing an infection, before immunity to challenge can be established (Fitzgerald, 1968). However evidence from irradiation studies with E.tenella has suggested that its lifecycle is interrupted before the emergence of pathogenic forms of the parasite (Hein, 1963), but even in this case slight clinical effects were seen in chickens after two inoculations with irradiated oocysts implying that not all the oocysts were altered enough to be non-pathogenic.

In the mouse studies in Chapter 5 oocysts irradiated with 5 krad were found to be capable of causing chronic infection. In order to further test this observation ewes were inoculated during mid-pregnancy with oocysts treated in the same way. Only two out of the three ewes inoculated developed a pyrexia during the 18 days of monitoring, which suggests that a short lived parasitaemia took place, however they all developed anti-toxoplasma antibody. At lambing only one live lamb out of a possible four was produced. Furthermore all the stillbirths and the live lamb displayed histopathological evidence of Toxoplasma infection. Together with the mouse studies these results demonstrate that T.gondii oocysts irradiated with 5 krad retain the capacity to multiply and to change into tachyzoites and then bradyzoites. As a result of this they are able to induce both a parasitaemia and a chronic

infection. Due to the lesions in both the placentae and foetuses it is clear that they also retain their pathogenicity.

CONCLUSIONS

Subcutaneous inoculation of sheep with as few as 20 M1 strain oocysts can induce pyrexia, seroconversion and protection against subsequent challenge. These effects were also found after infection with irradiated oocysts. Oocysts treated with 5 krad irradiation were capable of crossing the placenta, following inoculation at mid gestation. Their pathogenicity for the foetus was also comparable with that of untreated oocysts. While not conclusive this data further suggests that irradiation kills a proportion of oocysts whilst leaving the rest unaltered.

Table 6.1 Design of Experiment 1: Subcutaneous inoculation of untreated and irradiated Toxoplasma gondii oocysts and subsequent challenge.

GROUP	NO.OF SHEEP	DOSE OF OOCYSTS (IRRADIATION KRAD) DAY 0	CHALLENGE OF OOCYSTS DAY 56
1	2	NIL (-)	2000
2	2	20 (NIL)	2000
3	2	200 (NIL)	2000
4	2	2000 (NIL)	2000
5	2	2000 (5)	2000
6	2	2000 (10)	2000

Table 6.2 Design of Experiment 2: Subcutaneous
inoculation of Toxoplasma gondii
irradiated oocysts during mid-pregnancy

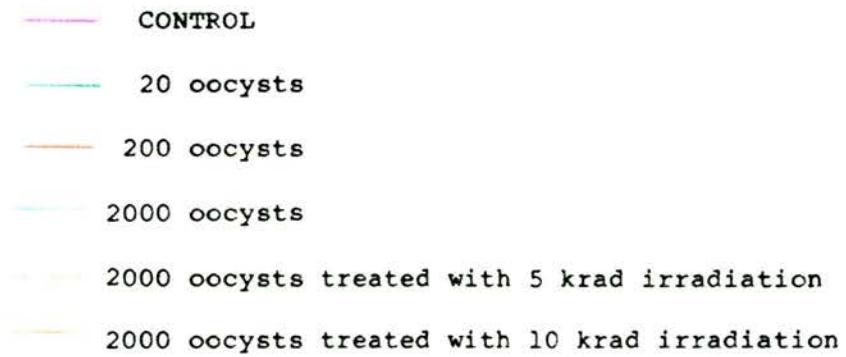
NO. OF SHEEP	DOSE OF OOCYSTS (IRRADIATION KRAD) 80 DAYS OF GESTATION
3	2000 (5)

TABLE 6.3 Outcome of pregnancies and histological evidence of
Toxoplasma infection in the foetuses.

EWE	NO. LAMBS	LIVE	STILLBORN	BRAIN LESIONS OF TOXOPLASMOSIS	PLACENTAL LESIONS OF TOXOPLASMOSIS
1	1	1	-	ND+	YES
2	2	0	2	YES	YES
3	1	0	1	YES	YES

+ ND - NOT DONE

FIGURE 6.1 Mean rectal temperatures of sheep given untreated and irradiated Toxoplasma gondii oocysts.



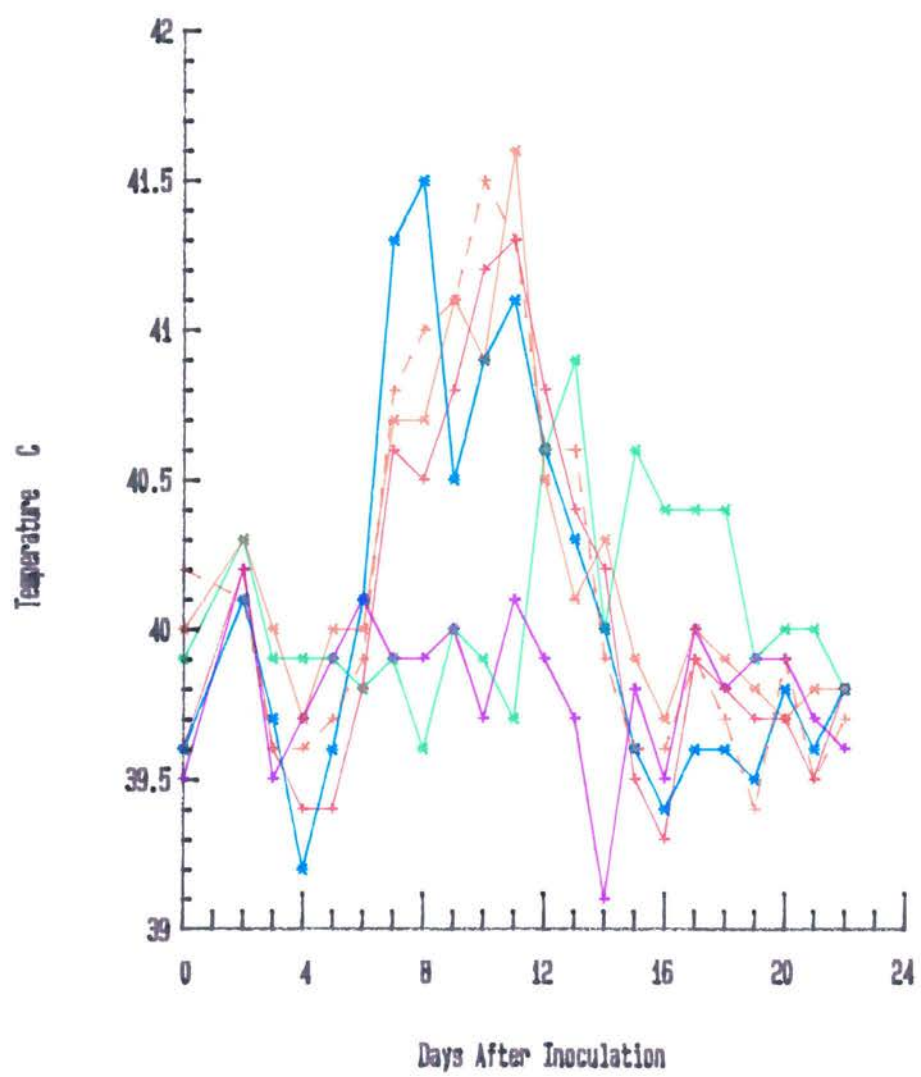
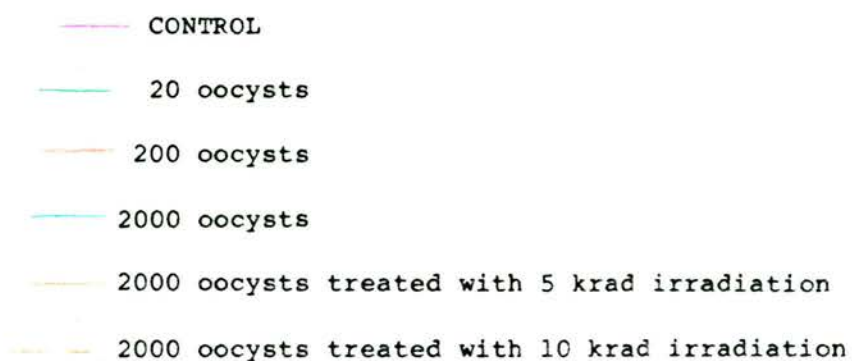


FIGURE 6.2 IgG antibody against Toxoplasma (mean of per cent O.D.) detected in serum of sheep given untreated and irradiated oocysts.



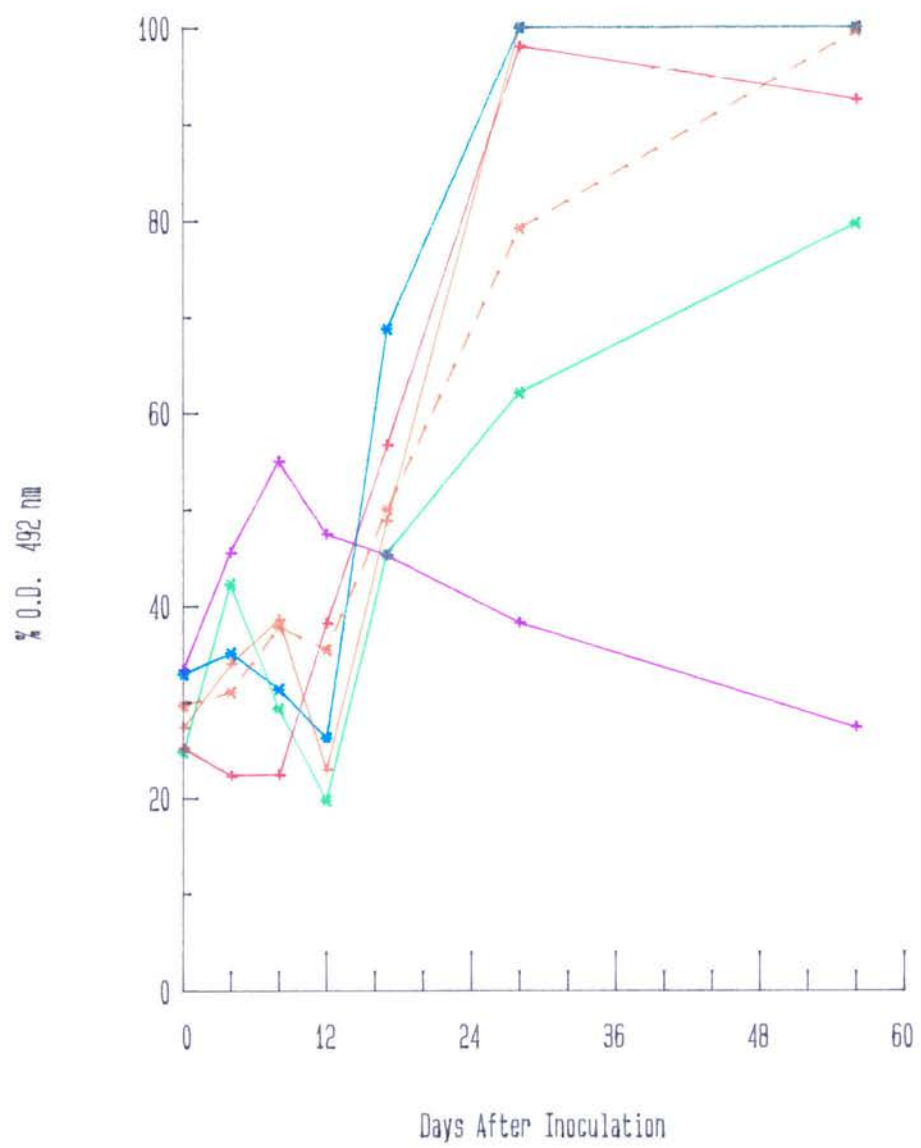


FIGURE 6.3 Mean rectal temperature of sheep
after challenge infection with
Toxoplasma gondii oocysts.

— nil/2000 oocysts
— pooled results for all sheep that
received a challenge infection.

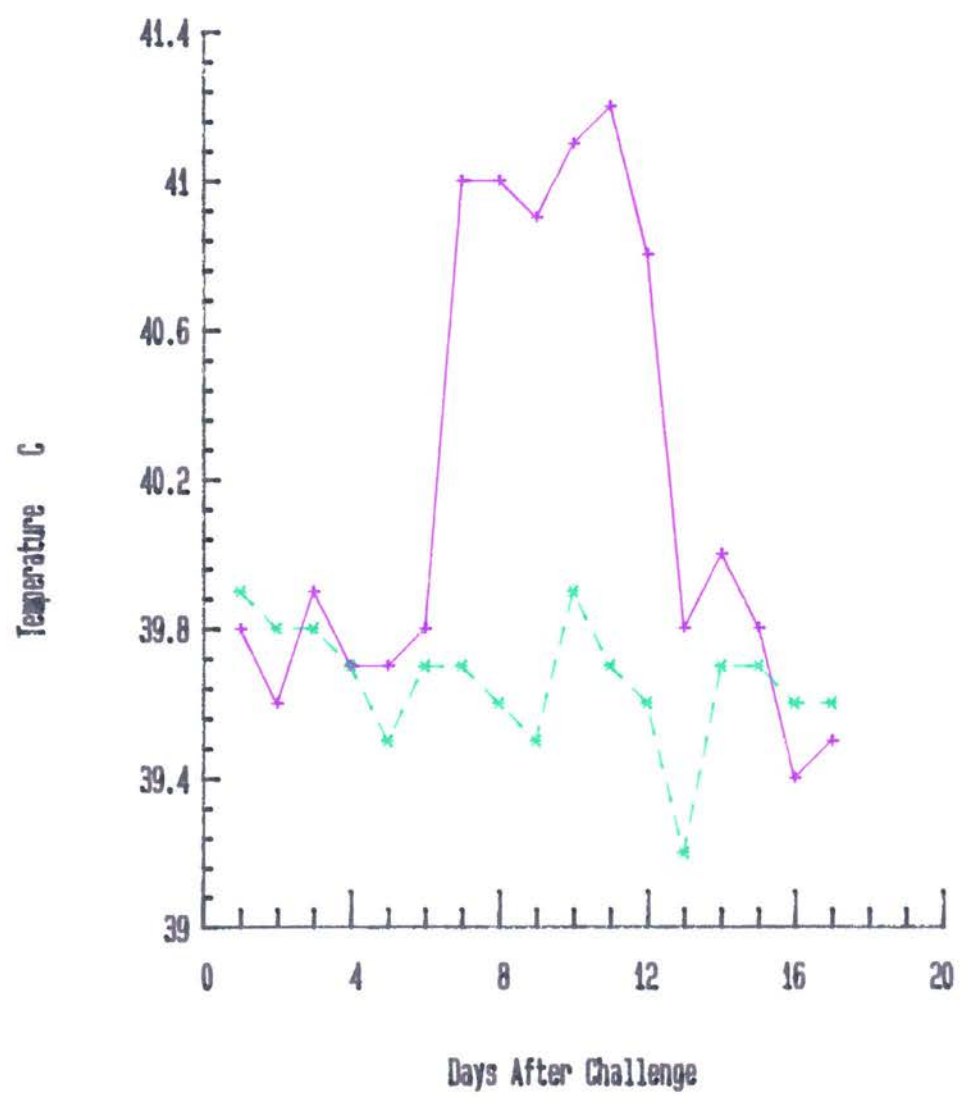


FIGURE 6.4 IgG antibody against Toxoplasma
(mean of per cent O.D.) detected in
serum of sheep challenged with
Toxoplasma oocysts.

— nil/2000 oocysts
— 20/2000 oocysts
— 200/2000 oocysts
— 2000/2000 oocysts
— 2000 (5 krad)/2000 oocysts
— 2000 (10 krad)/2000 oocysts

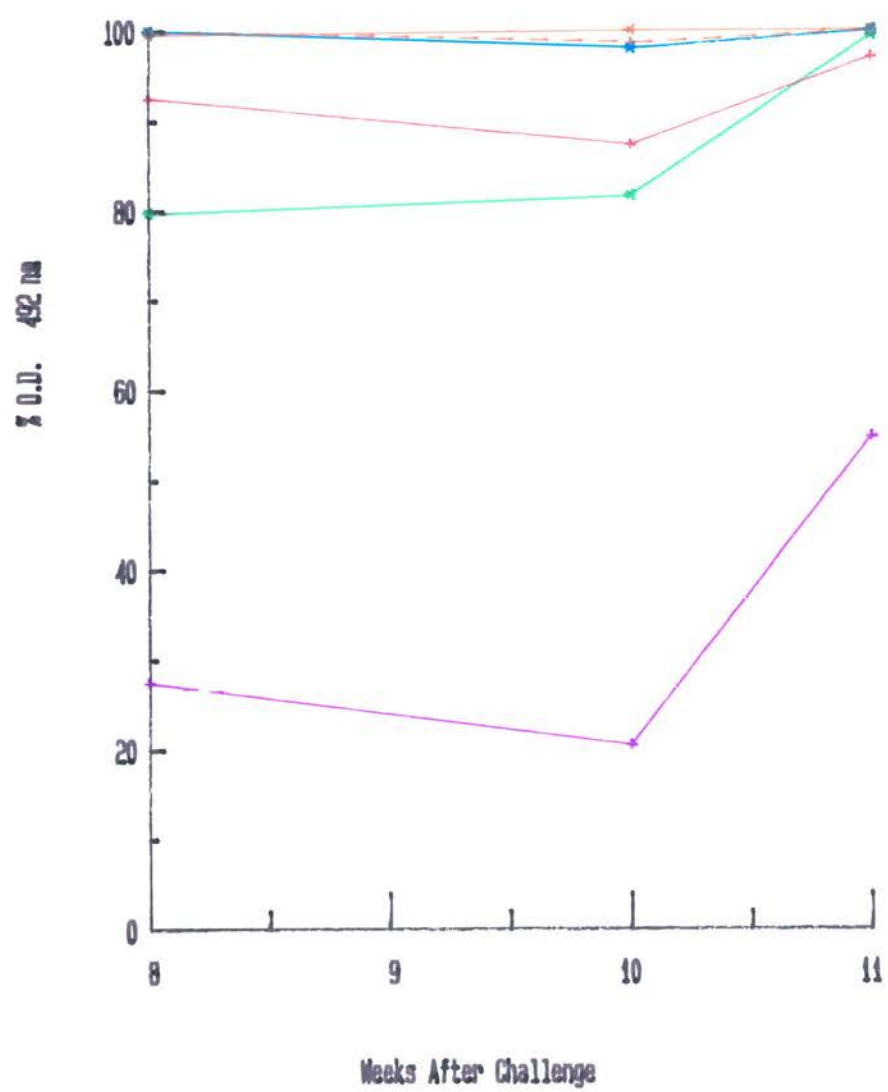


FIGURE 6.5 Rectal temperature response after inoculation with 2000 oocysts irradiated with 5 krad.

— Responders
— Non responder

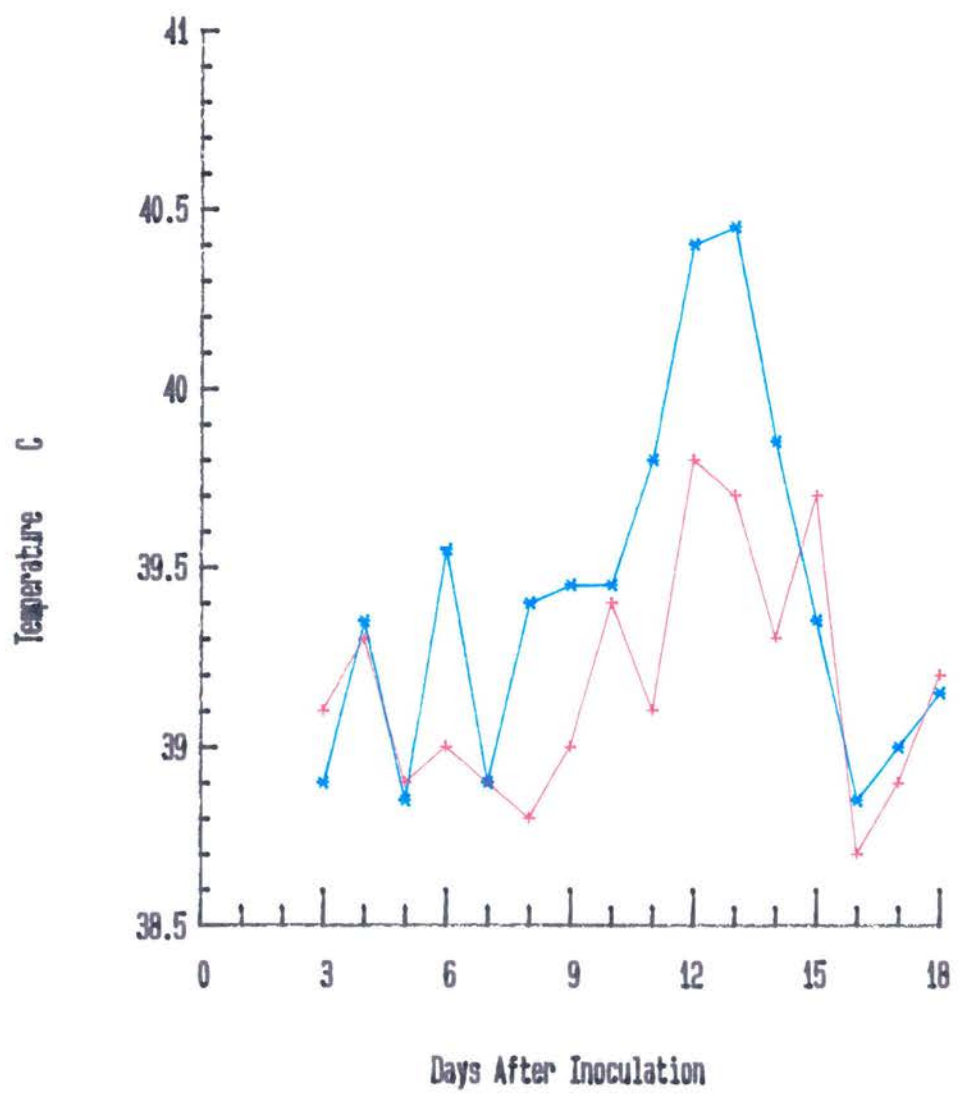
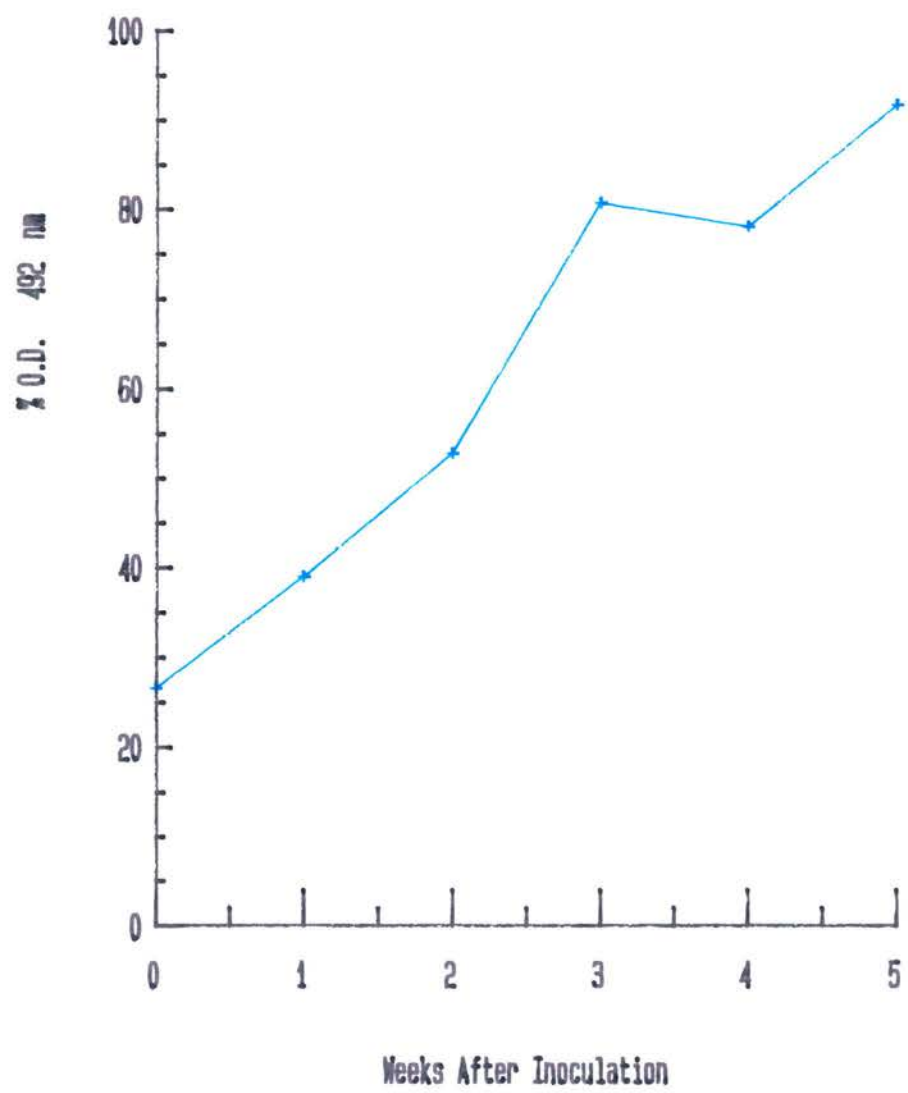


FIGURE 6.6 Anti-toxoplasma IgG antibody detected in
the sera after inoculation with 2000
oocysts irradiated with 5 krad.



CHAPTER 7 Changes in ovine popliteal efferent lymph following
infection with Toxoplasma gondii.

INTRODUCTION

Ever since T.gondii has been recognised as a cause of ovine abortion there has been considerable interest in the immune response of sheep to infection. Most studies have concentrated on serological aspects of immunity and data from both experimental and naturally infected animals show that a substantial antibody response is induced (Hartley, 1964; Blewett et al, 1982; Miller et al, 1982; Buxton and Finlayson, 1986). More detailed studies by Blewett and coworkers (1983) demonstrated that an IgM response could initially be detected 10 days after infection by an IHA test and this gradually decreased and was replaced by IgG. The only indications that an ovine cell mediated response occurs comes from histopathological studies with both adult and foetal animals (Koestner and Cole, 1961; Sharma and Gautam, 1978; Buxton and Finlayson, 1986).

More information is required on the sequence of immunological events following Toxoplasma infection in sheep in order to obtain a better understanding of the host parasite relationship. The chronic cannulation of an efferent lymphatic duct, which drains a single lymph node, permits the study of cells leaving a lymph node following challenge with an antigen, throughout an immune response. This provides an excellent tool for the dissection of the ovine immune response to T.gondii infection.

The popliteal lymph node drains the tissue near the hoof and hock in sheep and this is a convenient area in which to inject antigens by the s.c. route. Hall and Morris (1962) were the first to describe the technique for cannulation of the popliteal efferent

lymphatic and since then several workers have used this method to monitor the immune response to viral, bacterial and cellular antigens.

Buxton and coworkers (1981) used this technique to carry out preliminary studies on Toxoplasma infection in sheep. They found that lymph, obtained from sheep experiencing a primary infection with Toxoplasma, contained live organisms whereas it was only on very rare occasions that they could be detected in the lymph from naturally immune animals.

The aim therefore, was to study the changes in cell output from the cannulated efferent lymphatic following both primary and secondary infection with T.gondii.

Initial training and assistance with the cannulation procedures was kindly given by Dr H P R Miller.

EXPERIMENTAL PROCEDURE

Suspensions of 100 T.gondii tissue cysts per ml were prepared from infected mouse brain (see Chapter 2) whilst control inoculum was prepared in the same manner from the brains of normal uninfected mice.

Fourteen sheep of either Cheviot, Merino or Scottish Blackface breeds were employed. One popliteal efferent lymphatic from each animal was cannulated as described in Chapter 2. After the operation sheep were confined in metabolism crates and given water and feed concentrates ad libitum. Twelve of the sheep had no detectable anti-toxoplasma antibody by IgG-ELISA whilst the

remaining two sheep had naturally acquired antibody. Seven of the susceptible animals and the two with naturally acquired antibody (Group 1 and 2 respectively) were each given 100 T.gondii tissue cysts by s.c. inoculation on the lateral aspect of the cannulated leg below the hock joint. The remaining five (Group 3) were injected with the control inoculum in the same manner.

Lymph was collected and examined as described in Chapter 2. Sheep were monitored daily for clinical signs of illness, including a pyrexia and they were killed by i.v. injection of pentobarbitone sodium (300 mg) after the lymph had ceased to flow. The popliteal and internal iliac lymph nodes from both sides of the animal were removed and treated as described in Chapter 2.

RESULTS

CLINICAL SYMPTOMS

All the sheep experiencing a primary infection developed a pyrexia by six days after the infection. Neither the control animals nor those with naturally acquired antibody developed a temperature after inoculation.

CELL OUTPUT FROM THE POPLITEAL EFFERENT LYMPH DUCT

Total cell output

Following inoculation with T.gondii tissue cysts, sheep in group 1 (naive challenge) showed a steady rise in total cell output (Fig 7.1) and from six to 10 days after infection the greatest number of cells were found in the lymph. By day 16 this had decreased to the mean preinoculation level. The total cell output of immune animals which were challenged (Group 2) rose until day 5 when it was twice

the value of the preinoculation counts. The average number of cells in the efferent lymph from control sheep (Group 3) reached a peak by the fifth day after inoculation when it showed a threefold increase over preinoculation levels.

Lymphoblast Output

The percentage of lymphoblast cells, defined as large cells with irregular nuclei and basophilic cytoplasm (Fig 7.2), present in the lymph of sheep in group 1 increased throughout the course of infection (Fig 7.3). At their peak response on day 11 lymphoblasts accounted for 50.3 per cent of the total cell output while in contrast, the response in group 3 reached a peak of 13.9 per cent eight days after inoculation. It was shown by Student's T - test that the lymphoblast responses in group 1 on days 9,10 and 11 were significantly different from those in the control sheep. The peak of the lymphoblast cell response in group 2 (27.5 per cent) occurred four days after inoculation.

Immunoglobulin Containing Cells

IgM

Data on the percentage of cells which contained IgM was obtained from four of the seven sheep in group 1 (Fig 7.4). Analysis of this data revealed that three of these animals reacted in a similar manner, with a small proportion of the lymphocytes with intracellular IgM and most had lymphoblast morphology. This response reached a peak seven days after infection when 6.5 per cent of the cells contained Ig M (Fig 7.5). Examination of the cells from the fourth animal in this group revealed that 19 per cent of the cells contained IgM two days after the infection and this proportion decreased until day 12 when a

gradual rise began which reached a peak 16 days after inoculation. The percentage of lymphocytes which contained IgM rose after the challenge in the immune sheep (Group 2) and by four days after inoculation 11.5 per cent of all lymphocytes were in this category, whereas in the control animals no more than 0.5 per cent of lymphocytes contained IgM.

IgG

As with the IgM results, three naive sheep from group 1 gave similar responses, with only low numbers of cells which contained IgG (Fig 7.6). The highest proportion of cells which had intracellular IgG was found 11 days after infection (Fig 7.7). The percentage of cells, in the fourth sheep in group 1, which contained IgG began to rise at day 5 and reached a peak eight days after infection. Thereafter there was a proportion of cells which contained IgG present in the lymph every day. By day 5, 10.75 per cent of all lymphocytes from immune sheep (Group 2) contained IgG. Lymph from control animals showed a slight increase in positively stained cells but the total number of cells that contained IgG never exceeded 3.5 per cent.

Antibody in Lymph

Specific IgG was first detected on day 11 in ewes that received a primary infection (Group 1) whilst in the immune animals (Group 2) anti-toxoplasma antibody was detected from day 1 and this rose daily after challenge (Fig 7.8). No anti-toxoplasma antibody was detected in control animals at any time.

Necropsy

Efferent lymph flowed for a mean period of 12 days in group 1, 10 days in group 2 and nine days in the controls (Table 7.1). In group 1 popliteal and internal iliac lymph nodes from the cannulated and injected side of the animals were enlarged and measured between 25 and 30 mm long and approximately 20 mm wide. The contralateral lymph nodes were similar in size to those in control animals and measured 10-15 mm by on average 7.5 mm. The lymph nodes from animals in group 2 were intermediate in size.

Histopathology of Lymph Nodes

Examination of the popliteal lymph nodes which drained the site of infection in naive animals revealed several changes. The capsule was thickened and there were mononuclear cells present both within and outside the capsule. These cells were predominantly lymphocytes, however occasional macrophages were seen. Both primary and secondary follicles were present in the cortex (Fig. 7.9), but the extent of the development varied between animals and ranged from one to three layers. The paracortex of the lymph nodes did not appear to be significantly expanded, whereas both the medullary sinuses and cords were enlarged and frequently contained a large number of lymphocytes and macrophages (Fig. 7.10). Similar changes were seen in the internal iliac and contralateral popliteal lymph nodes which were removed from the infected animals. Lymph nodes obtained from both the immune and control animals showed less dramatic changes.

Examination of the lymph nodes from infected animals revealed small numbers of cells which contained either IgM (Fig. 7.11) or IgG (Fig. 7.12) in the medulla and on occasions intracellular IgM

was seen in cells in the follicles (Fig 7.13). The other lymph nodes which were removed from the infected animals and the lymph nodes from immune animals had a similar distribution of immunoglobulin containing cells.

DISCUSSION

The local immune response to both primary and secondary infection with T.gondii tissue cysts was monitored by cannulation of popliteal efferent lymphatics. Toxoplasma oocysts were unavailable at the time of experimentation therefore tissue cysts obtained from infected mouse brains were used as the inoculum.

As previously stated (Chapter 4; Blewett et al, 1982; Miller et al, 1982) only animals experiencing a primary infection developed a pyrexia. No other clinical signs were apparent.

The variation in both flow rate and cell output from individual cannulated efferent lymphatics was large, however the underlying trends were consistent. The control animals which were given uninfected mouse brain responded with a sharp increase in cell output three days after inoculation. This presumably reflects the cell recruitment phase seen with other antigens and is to be expected due to the antigenic nature of the homogenised material inoculated. This phase was short lived and only a slight increase in the number of proliferating cells in the lymph were found. The percentage of cells which contained IgM or IgG remained low throughout the experiment.

The cell output from primary, infected sheep increased five-fold six days after injection of tissue cysts and this output was

sustained for several days. The proportion of these cells which had blast cell characteristics increased dramatically over this period, however in three out of the four animals tested only a small percentage of the cells contained either IgM or IgG. Lymph from the fourth animal in group 1 contained a higher proportion of cells with cytoplasmic IgM or IgG and the IgM response was also biphasic. The difference was not in the proportion of cells which had blast cell characteristics but in the number of these cells which contained Ig. Why this animal responded differently is not known, however an explanation can be offered for one aspect of the response. The lymph from this animal flowed for 24 days, considerably longer than in the other animals, therefore the biphasic IgM response, which presumably reflects the appearance of new antigens may have occurred with the other animals if the lymph had flowed for long enough. When animals naturally immune to T.gondii were challenged with tissue cysts, the lymphoblast response was less dramatic, but more rapid in onset, than those animals that received a primary infection and its peak coincided with the peak of the Ig-containing cell response, when more than 50 per cent of all lymphoblasts contained either IgM or IgG.

Several other workers have used chronic efferent lymphatic cannulations to monitor changes in cellular components after antigenic stimulation. Comparison of these published results with the data reported here show several similarities. The flow rate, cell output and cellular composition of the lymph before challenge was comparable with earlier studies (Heath, Lascelles and Morris, 1962; Smith, McIntosh and Morris, 1970). In addition there were no plasma cells present in the efferent lymph after antigenic

stimulation, a finding previously reported following bacterial and viral challenge (Hall, Morris, Moreno and Bessis, 1967; Murphy, Hay, Morris and Bessis, 1971; English, Morris and Adams, 1976). Although in the present study, an increase in cell output of lymphocytes had started two days after injection, it did not reach a peak until day 6 and remained high for several days afterwards. The reason for the prolonged nature of this response is not clear but it may be explained by the characteristics of the Toxoplasma organism. At various stages after infection it would be expected that either bradyzoites, or the fast multiplying tachyzoites, would be the predominant form of Toxoplasma present at any one time. Thus it is possible that any differences in the antigenic structure between these two stages could result in the recruitment and proliferation of lymphocytes with different specificities.

Unlike the response to bacterial and viral challenge (Hall et al, 1967; Murphy et al, 1971; English et al, 1976) only a few cells which contained Ig were detected in the efferent lymph from animals given a primary infection. However, in studies by other workers when allogenic lymphocytes were used as the antigen in cannulation experiments the majority of cells in the efferent lymph did not contain Ig (Hay and Cahill, 1981). As allogenic lymphocytes are known to induce a T cell response it is possible that the ovine primary response to Toxoplasma is also a predominantly cellular one. Although some of the Ig negative cells may have been null cells (Fahey 1980; Outteridge, Fahey and Lee, 1981) it seems likely that the majority of them were T cells. At the time of experimentation no satisfactory method of identifying ovine T cells was available and further characterisation of these cells was not

therefore possible. Immunohistological examination of the lymph nodes from these infected animals revealed follicular development and a few cells that contained Ig. However since these lymph nodes were removed after the lymph had ceased to flow and therefore at different times after challenge, it would be expected to find various stages in the immune response to Toxoplasma, but even so it was difficult to obtain a coherent picture.

As previously stated in Chapter 1, T cells appear to play a central role in the immune response to Toxoplasma infection both in Man and mice. The early studies by Frenkel (1967) clearly demonstrated that protection against Toxoplasma could be obtained by the transfer of immune lymphocytes and not by immune serum. Further evidence for the importance of T cells comes from studies on athymic nude mice which are unable to mount an immune response, either cellular or humoral, to Toxoplasma gondii thus leading to their eventual death (Lindberg and Frenkel, 1977; Buxton, 1980).

The appearance of lymphoblast cells occurred sooner in the naturally immune animals than in those experiencing a primary infection, a finding previously reported for bacterial and viral antigens. However the more rapid onset of this response is also normally accompanied by an increase in magnitude. This was not the case in animals used in the Toxoplasma study. In a previous study, with a bacterial antigen, animals were infected either systemically or in the draining area of the lymph node to be cannulated. They were subsequently cannulated and challenged locally (English et al, 1976). The data revealed that in animals infected systemically, although the onset of the response was comparable with the animals that had received two local inoculations, only 27 per cent of the

cells were lymphoblasts compared with 70 per cent in the latter group. It is possible that a similar situation occurred in this study since the immune animals had almost certainly become infected following ingestion of oocysts in the field. Although serum levels of anti-toxoplasma antibody were high in the animals from Group 2 before challenge, antibody could only be detected in the lymph from the first day after inoculation. This lends further support to the suggestion that the initial infection was systemic.

From the data it appears that there are differences in the cell types present during primary and secondary infection with Toxoplasma. It is possible that during the secondary infection specific antibody, which is rapidly produced, is playing an important role. English and his coworkers (1976) found considerable numbers of lymphoblasts which contained Ig after a secondary challenge with *Salmonella* and demonstrated that they were secreting specific antibody against the organism. These workers suggested that this early production of antibody would be beneficial since it would flood the the extravascular spaces and the blood stream, enhancing the early clearance of antigen. It is known that following a primary infection with T.gondii, efferent lymph from the infected node can contain live organisms, while lymph from immune animals with rare exceptions does not (Buxton et al, 1981). The virtual absence of infection in efferent lymph from immune animals may be explained by the presence of specific antibody at the time of challenge. This antibody could opsonise the parasites, leading to increased phagocytosis and intracellular killing by macrophages, as has been reported in other species (Jones et al, 1975; Shirahata et al, 1976). This mechanism could not occur as

rapidly in a primary infection, since IgG antibody is not detected until day 11, allowing the multiplication of T.gondii to proceed unhindered in the initial stages of infection. As the serological test used in this study did not detect IgM antibody, its presence in lymph soon after infection cannot be excluded. However, IgM antibody to Toxoplasma has not been detected in the sera of experimentally infected sheep before day 10 (Blewett et al, 1983), so it seems unlikely that it was present in the lymph immediately after infection.

CONCLUSIONS

Toxoplasma induces a substantial lymphoblast response in the efferent lymph from popliteal lymph nodes. The predominant cell type during primary infection is Ig negative and presumably represents a population of T cells, while during secondary infection more Ig containing cells are present. This may reflect different mechanisms whereby opsonisation by antibody may play an important role during secondary infection.

TABLE 7.1 Duration of lymph flow from ovine cannulated popliteal efferent lymphatics.

SHEEP	GROUP	NO.OF DAYS LYMPH FLOWING
1	1	5
2	1	9
3	1	10
4	1	10
5	1	14
6	1	15
7	1	24
8	2	6
9	2	15
10	3	6
11	3	9
12	3	9
13	3	11
14	3	11

FIGURE 7.1 The mean cell output (+ standard errors)
from the cannulated efferent lymphatic
ducts of sheep inoculated with one
hundred T.gondii tissue cysts;
susceptible sheep — immune sheep —
or control inoculum — .

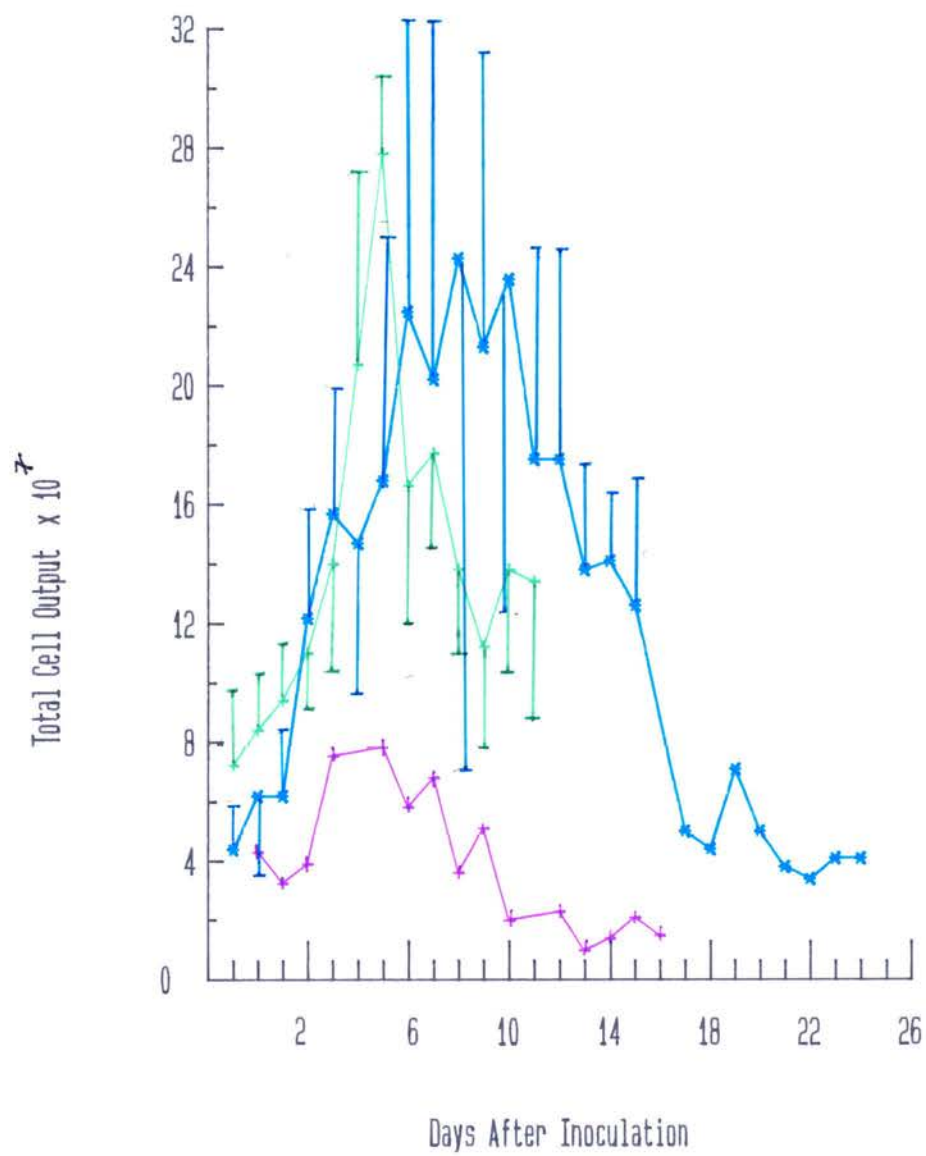


FIGURE 7.2 Cytocentrifuge preparation of cells
from the popliteal efferent lymph
stained with Leishman's. Both small
lymphocytes and lymphoblasts are
present.

x 1000

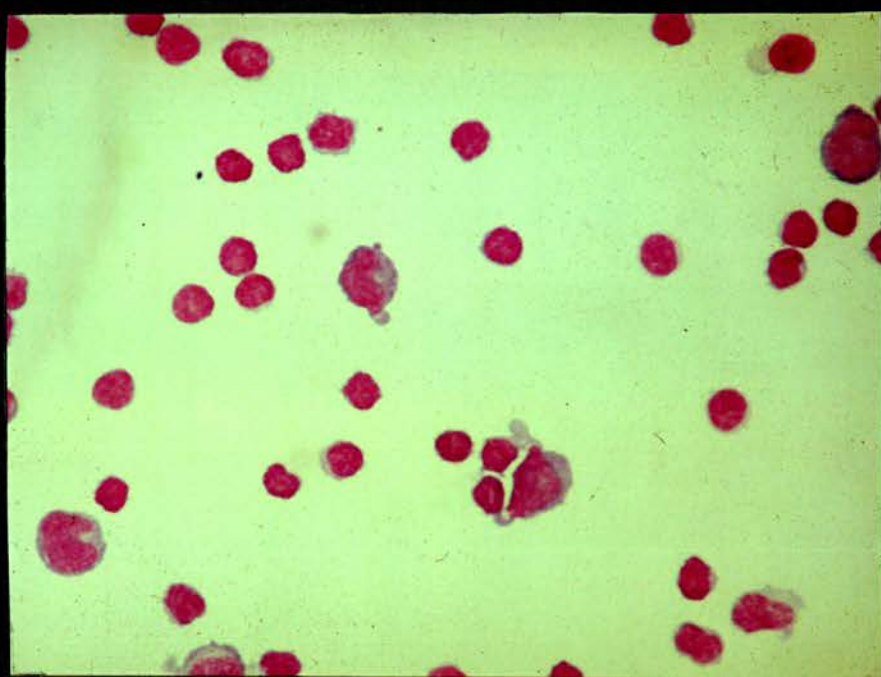


FIGURE 7.3 The mean percentage lymphoblast output
 (+ standard errors) from the cannulated
 efferent lymphatic ducts of sheep
 inoculated with one hundred T.gondii
 tissue cysts; susceptible sheep,
 — immune sheep — or control
 inoculum . —

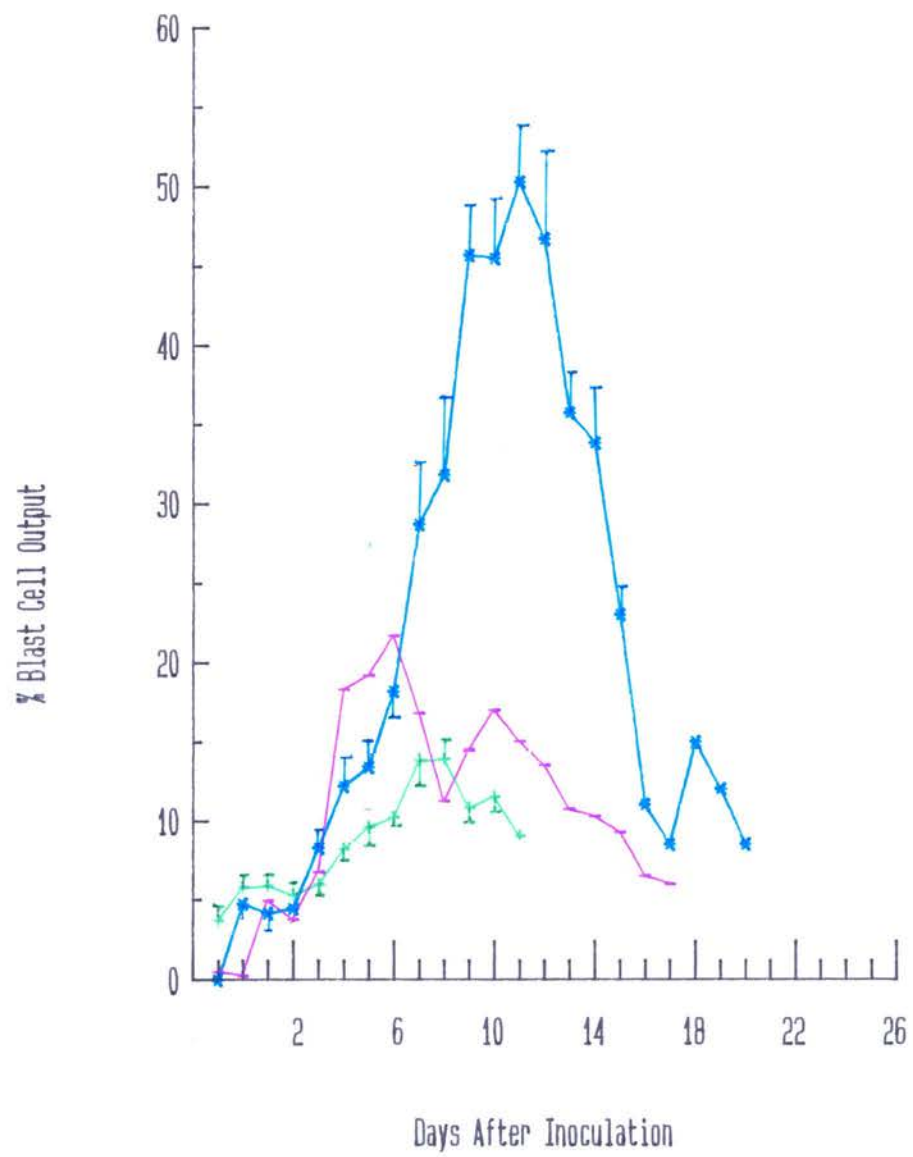


FIGURE 7.4 Cytocentrifuge preparation of cells
from popliteal efferent lymph. Cells
which contain IgM are detected by
an α -ovine IgM conjugated to HRP.

x 1000

FIGURE 7.5 Cytocentrifuge preparation of cells
from popliteal efferent lymph. Cells
which contain IgG are detected by
anti-ovine IgG conjugated to HRP.

x 1000

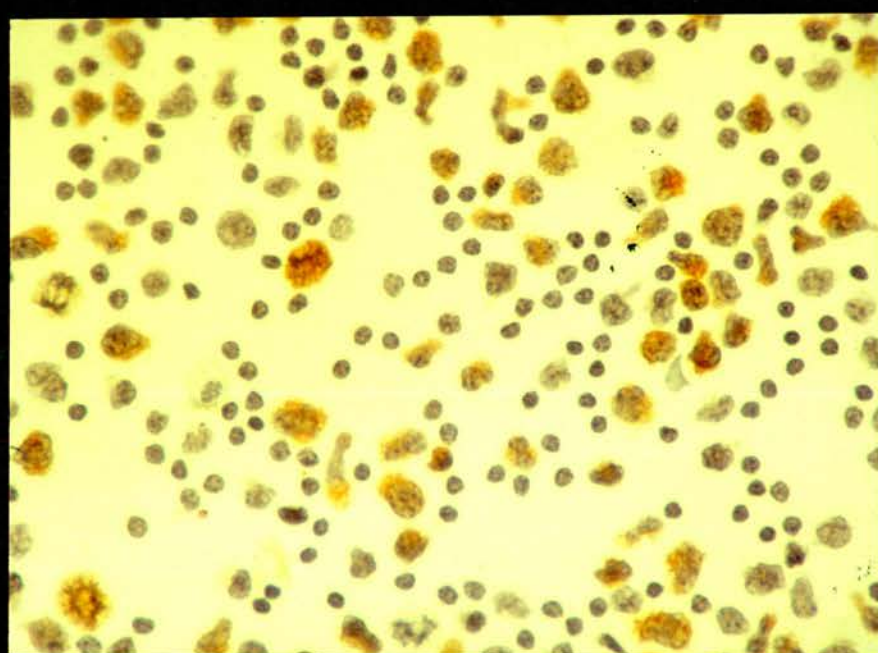


FIGURE 7.6 The mean percentage of IgM-containing
 cells in the efferent lymph of sheep
 inoculated with T.gondii tissue cysts;
 susceptible sheep low responders ———
 high responder ——— immune sheep ———
 or control inoculum ——— .

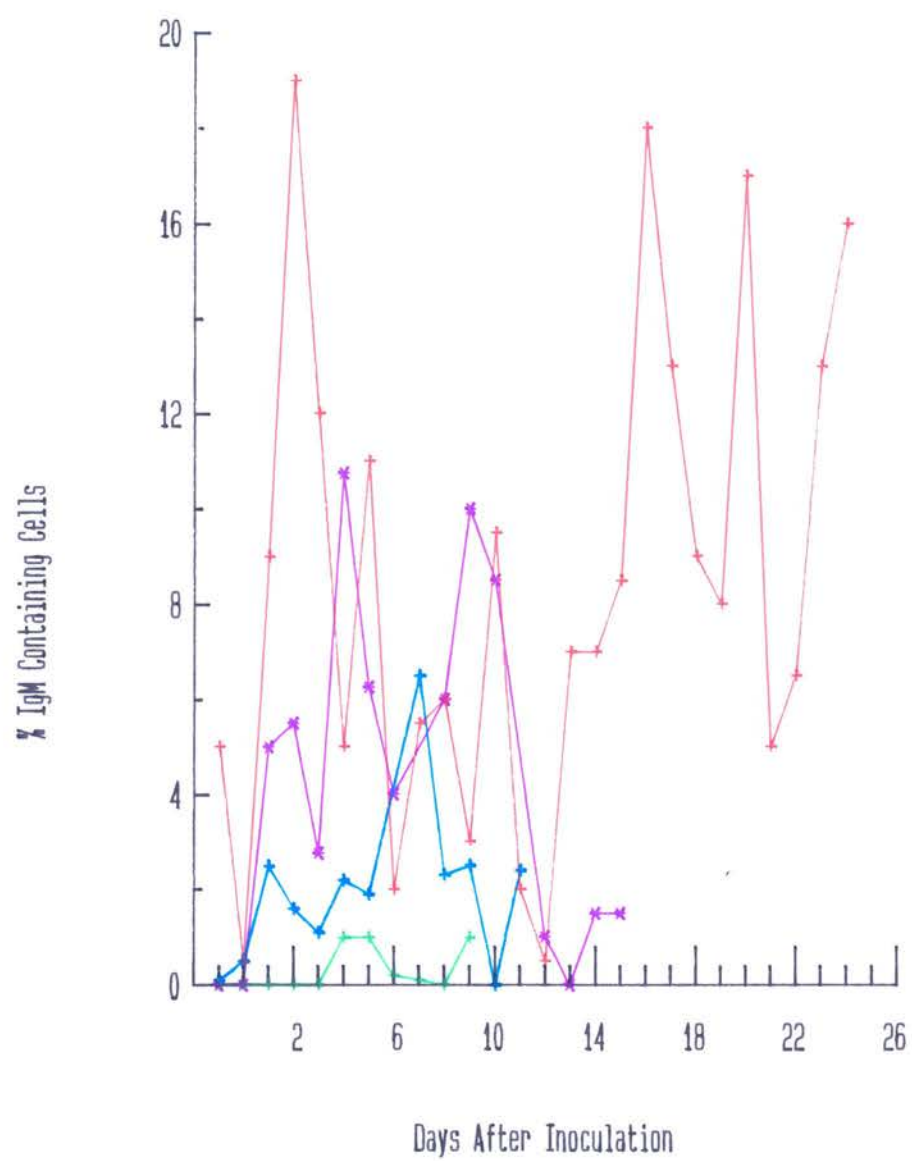


FIGURE 7.7 The mean percentage of IgG-containing cells in the efferent lymph of sheep inoculated with T.gondii tissue cysts; susceptible sheep low responders — high responder, — immune sheep — or control inoculum — .

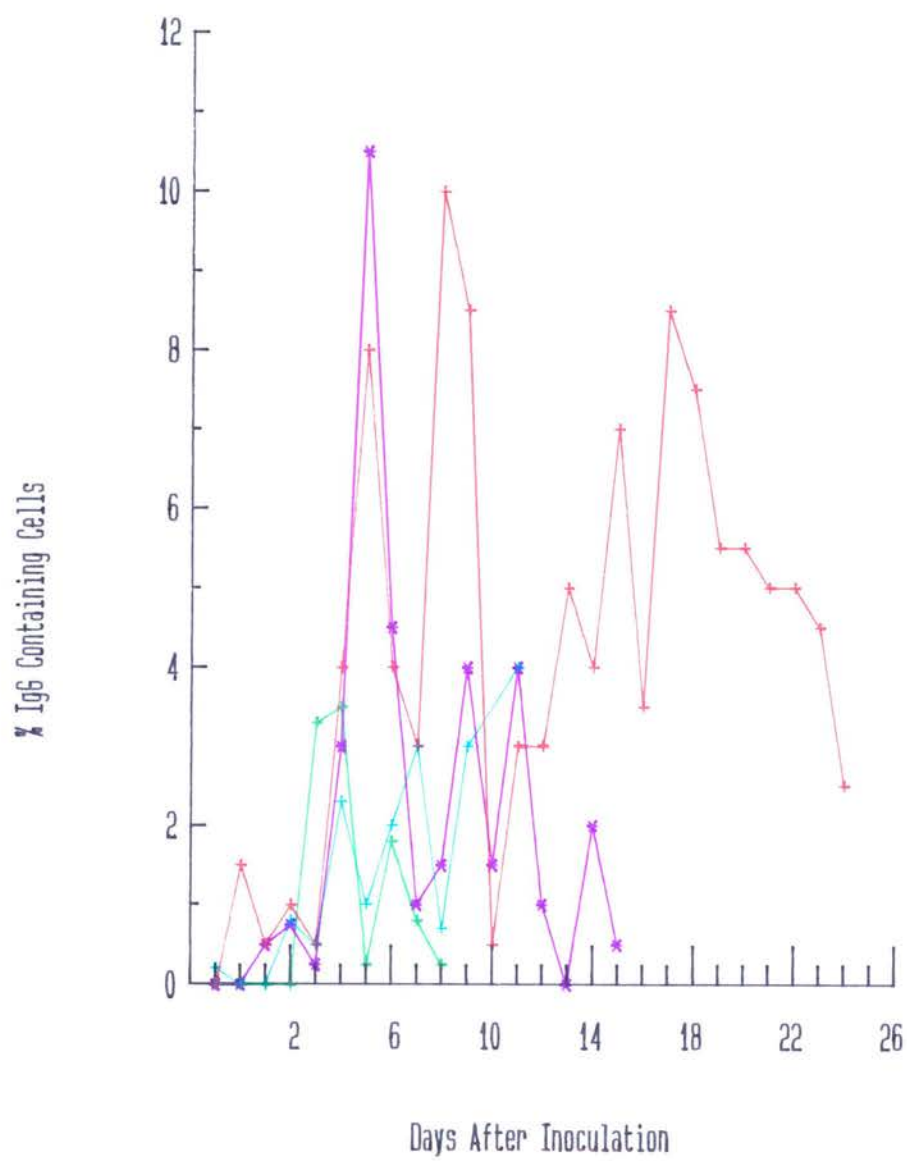


FIGURE 7.8 Anti-toxoplasma IgG in the efferent
lymph of susceptible — or immune
sheep — following inoculation
with T.gondii tissue cysts, or in
susceptible sheep after control
inoculum.

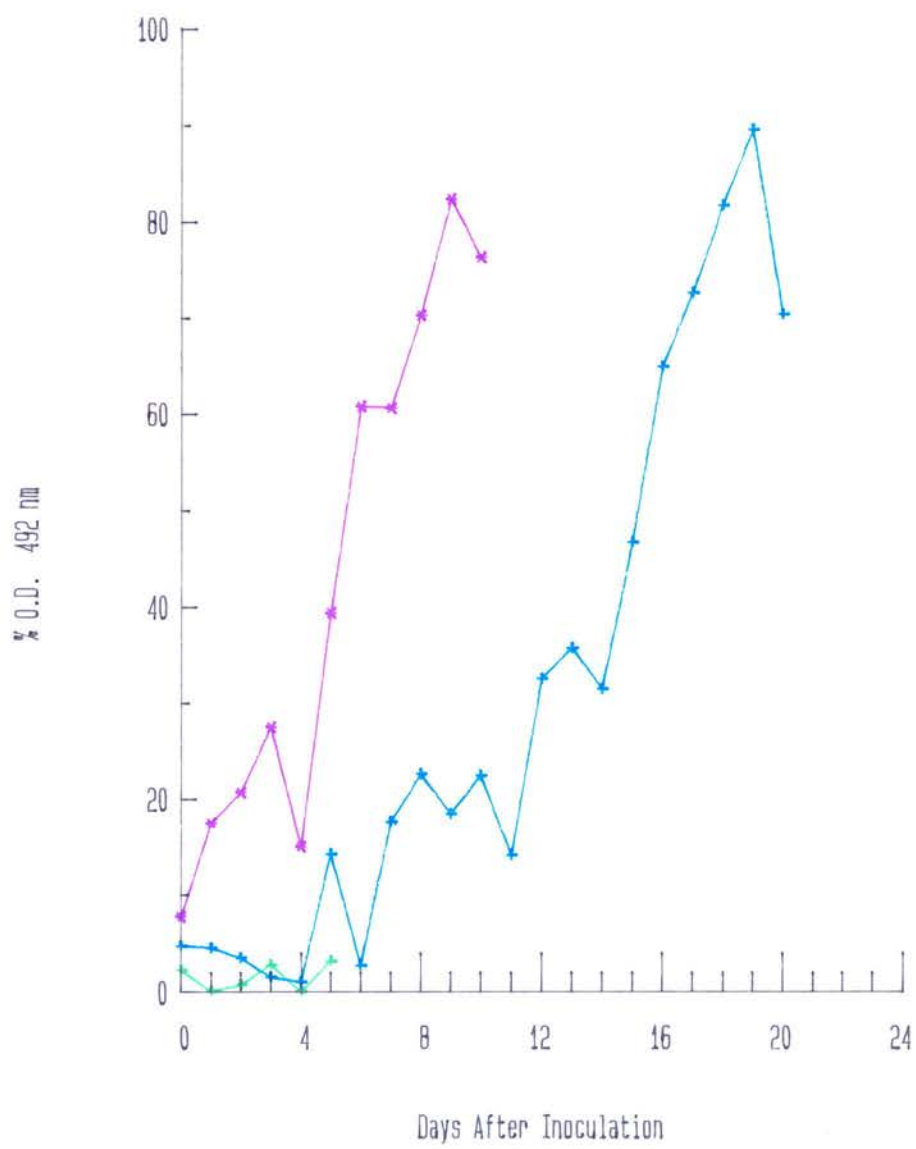


FIGURE 7.9 Cortical area of popliteal lymph node
draining the infection site
demonstrating follicular development.

H & E x 600

FIGURE 7.10 Medulla of popliteal lymph node
draining the infection site
demonstrating enlarged medullary
sinuses and cords.

H & E x 600

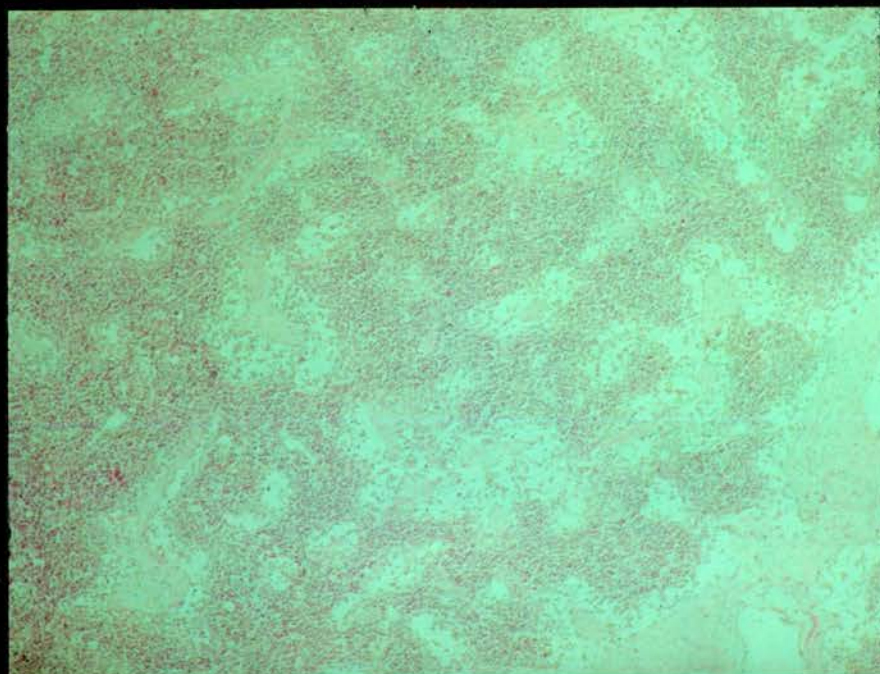
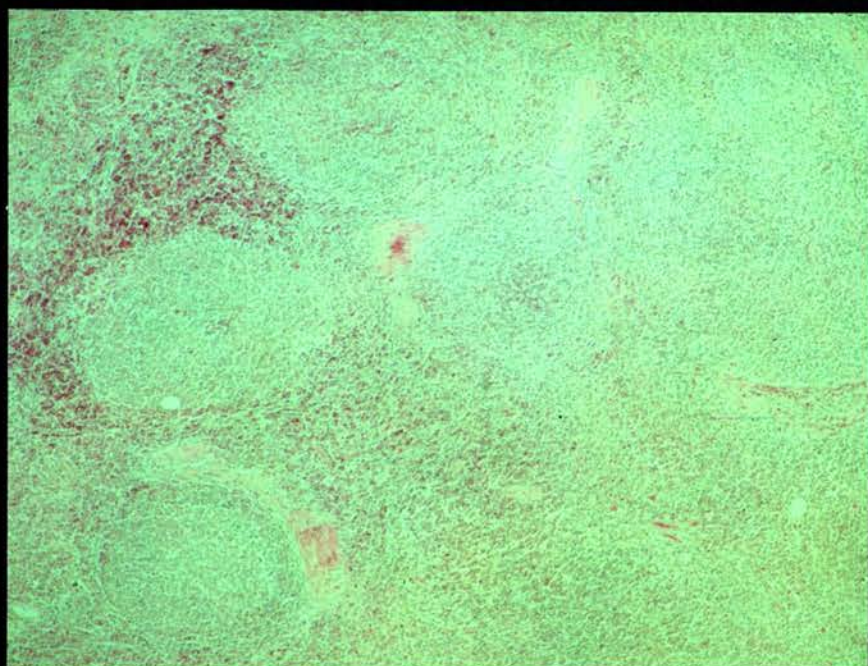


FIGURE 7.11 Ig M containing cells in medulla of popliteal lymph node after Toxoplasma infection. Rabbit anti-sheep conjugated to HRP was used.

x 600

FIGURE 7.12 Ig G containing cells in medulla of popliteal lymph node after Toxoplasma infection. Goat antisheep conjugated to HRP was used.

x 600

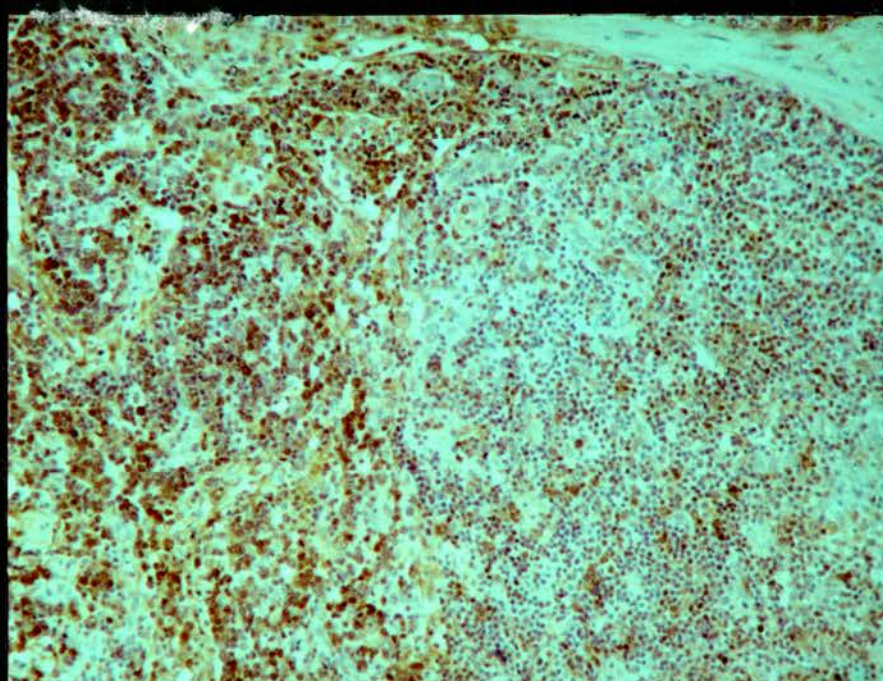
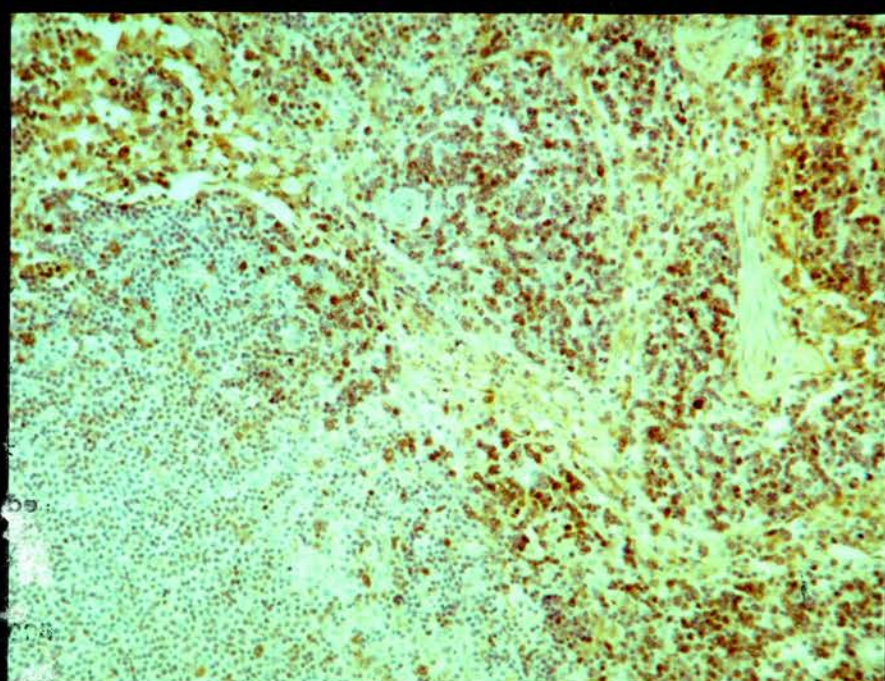
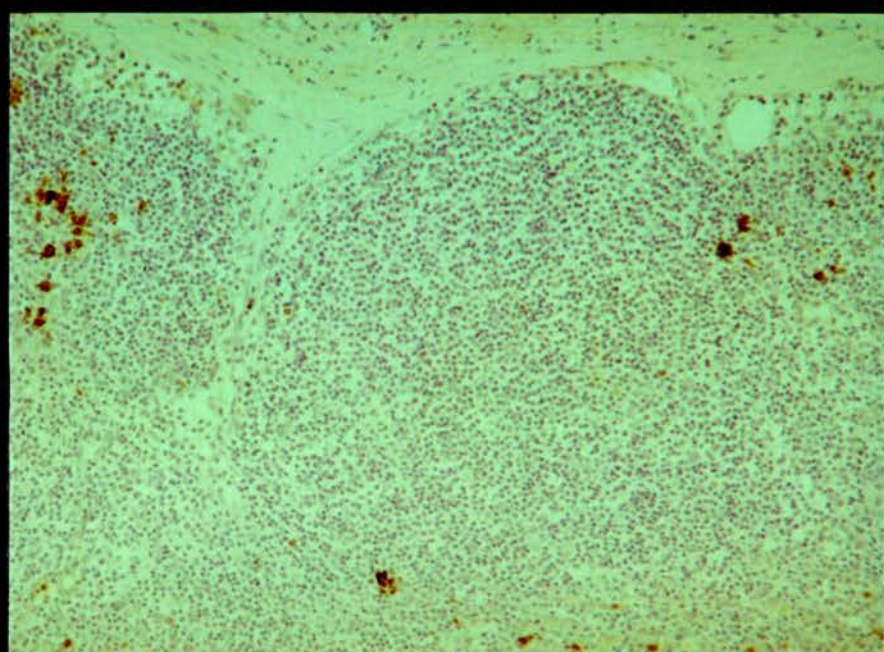


FIGURE 7.13 Ig M containing cells within follicles
of popliteal lymph node after
Toxoplasma infection. Rabbit antisheep
conjugated to HRP was used.

x 600



CHAPTER 8 Capacity of ovine mammary gland macrophages to
phagocytose Toxoplasma gondii tachyzoites

INTRODUCTION

Evidence obtained from in vitro studies in both man and mice has shown that macrophages from Toxoplasma infected subjects, that is activated macrophages, have an enhanced capacity for the intracellular killing of the parasite when compared with cells from susceptible animals. Furthermore increased phagocytosis and intracellular killing by both activated and normal macrophages can occur due to opsonisation by specific antibodies (Remington et al, 1972; Anderson and Remington, 1974).

In sheep there are no published reports on the ability of macrophages to phagocytose protozoa or the extent, if any, to which specific antibody can increase the process. However Donachie, Burrells, Sutherland, Gilmour and Gilmour (1986) reported that specific anti-pasteurella antibody enhanced phagocytosis of Pasteurella haemolytica by ovine lung macrophages. It was therefore considered worthwhile to examine the role that anti-toxoplasma antibody might play in phagocytosis of T. gondii.

The most commonly used source of macrophages from mice is the peritoneal cavity, whereas in man, monocytes obtained from the peripheral blood are either used immediately or cultured for several days until macrophage characteristics appear. In sheep neither of these two sources yield suitable numbers of the required cells. Previous work at MRI has shown that the peritoneal cavity of sheep is a very poor source of macrophages. Furthermore attempts by myself to isolate monocytes from peripheral blood, by separation on a Ficoll gradient followed by either density gradient centrifugation or by adherence of the mononuclear cell population to plastic, gave low yields with heavy contamination by lymphocytes.

Alveolar macrophages are easily obtained by lavage of lungs removed from sacrificed animals and these cells have previously been used in phagocytosis assays (Donachie et al, 1986). The only practical source of ovine lungs is the abattoir, but this does not permit experimental infection of animals before harvesting the macrophages. An alternative source of cells is from the non-lactating mammary gland of ewes. It has been shown that if LPS is infused into the mammary sinus and the cells harvested six days later then around 70 per cent of the population have macrophage characteristics (McDowell et al, 1969). It was therefore decided to use this site as a source of macrophages.

The aims of this study were to determine whether the presence of anti-toxoplasma antibody could enhance the phagocytic capacity of ovine macrophages and if there was any discernible difference between the macrophages from immune and non-immune animals.

EXPERIMENTAL PROCEDURE

Six Greyface, non-lactating ewes with no detectable anti-toxoplasma antibody were used in this study. All the animals had healthy udders with no evidence of past disease. Three ewes were each inoculated s.c. with 100 Toxoplasma tissue cysts prepared from infected mouse brain as previously described (Chapter 7), whilst the other three were given control inoculum prepared from uninfected mouse brain (Table 8.1).

The rectal temperature of each animal was measured and recorded daily for the first 10 days. After a period of at least four weeks had passed, macrophages were harvested according to the technique described in Chapter 2 (Fig 8.1).

Heat inactivated sera, obtained either from a sheep with detectable anti-toxoplasma antibody (immune serum) or from gnotobiotic lambs born at MRI (gnotobiotic serum), were diluted by a factor of ten by the addition of medium. Live or formalin inactivated RH strain tachyzoites were incubated for 30 minutes at 37°C on a rotator, in the presence of immune serum (Treatment A); gnotobiotic serum (Treatment B); or medium alone (Treatment C) (Table 8.2). Subsequently the tachyzoites were added to duplicate wells of macrophage monolayers at a ratio of 10 : 1 and placed in an incubator set at 37°C, with five per cent CO₂. Slides were removed after 0, 30 and 60 minutes, then fixed and stained as described in Chapter 2. The number of intracellular tachyzoites were counted in a sample of 200 cells from each well.

To take account of individual animal variation, statistical analysis was performed on the mean result from each set of duplicate wells and analysis of variance was used.

RESULTS

LIVE TACHYZOITES

Time 0 minutes

Immediately after the addition of the tachyzoites, no intracellular organisms were detected.

Time 30 minutes

Approximately 20 per cent of the cells contained parasites (Fig 8.2) but there was no discernible difference between the results for macrophages from immune or non immune animals. The proportion of cells from all treatment groups which contained only

one organism ranged from 8.7 to 13 per cent, (Fig 8.5) whilst those cells which had two intracellular organisms made up between 2.7 and 5.4 per cent of the total population. Three or more organisms were found in a small proportion of cells in all of the treatment groups (1.3 - 6 per cent).

There was no statistically significant difference between any of the various treatments or between macrophages obtained from immune or susceptible animals, as assessed by analysis of variance.

Time 60 minutes

Between 6 and 16.9 per cent of cells examined contained one organism (Fig 8.6), whilst two tachyzoites were found in a smaller number of cells (4.5 - 11.3 per cent). The proportion of cells from immune animals which contained three or more parasites was small (1 - 6 per cent), (Fig 8.3) whereas in the population obtained from susceptible animals there was a considerable number of cells in this category (4.4-18.2 per cent). However, counts for macrophages exposed to tachyzoites alone were obtained from only one sheep.

There was no significant difference between any of the treatment groups by analysis of variance.

FORMALIN INACTIVATED TACHYZOITES

Time 0 minutes

No intracellular organisms were detected at time 0 in any of the macrophage monolayers.

Time 30 minutes

Up to 20 per cent of the cells contained intracellular parasites, (Fig 8.7), however there was no difference between

macrophages from immune or susceptible animals in any of the treatment groups. The proportion of cells incubated with either tachyzoites plus gnotobiotic serum or tachyzoites alone which contained one organism was similar and ranged from 2.3 to 3.4 per cent. After incubation with tachyzoites plus immune serum (Treatment A), 13.1 per cent of the cells from immune animals and 10 per cent of them from sheep given uninfected mouse brain contained one organism. These results were significantly greater than for the other two treatments ($P < 0.001$). Four to 5.5 per cent of the macrophages incubated with tachyzoites plus immune serum contained two organisms whilst 1.9 - 3.8 per cent contained three or more (two organisms: Treatment A vs B or C $p < 0.001$). A very small proportion of the cells incubated with tachyzoites only or, in the presence of gnotobiotic serum, had two intracellular organisms (0.1 - 1 per cent) whilst none contained three or more parasites after this incubation period.

Time 60 minutes

In cells incubated with tachyzoites plus immune serum, the proportion that contained one organism ranged from 12.8 to 14.9 per cent (Fig 8.8). Between six and eight per cent of macrophages incubated with tachyzoites alone or in the presence of gnotobiotic serum contained one parasite.

Two organisms were found in 6.3 per cent of the cells from immune animals given treatment A compared with nine per cent of macrophages from susceptible sheep and three or more tachyzoites were present in approximately seven per cent of cells in each group. Less than three per cent of the cells incubated with tachyzoites alone or with gnotobiotic serum had two intracellular

parasites. Three or more organisms were found in a very small proportion of the cells from these treatment groups (0.3 - 3.2 per cent). Statistical analysis: one organism - Treatment A vs B or C for macrophages from immune sheep, $P < 0.05$; Treatment A vs C for macrophages from uninfected animals, $P < 0.05$; Two organisms - for macrophages from immune animals Treatment A vs B, $P < 0.001$; vs C, $P < 0.01$.

DISCUSSION

The role of the macrophage in the ovine immune response to Toxoplasma infection does not appear to have received any attention until this study. Thus while it is well established that there is a humoral response to infection no functional immune studies have been performed. These experiments were carried out to assess both the capacity of ovine macrophages to phagocytose Toxoplasma tachyzoites and the ability of antibody to enhance the process.

Irrespective of whether live or killed tachyzoites were used in this system, less than 50 per cent of the macrophages had intracellular organisms, even after 60 minutes incubation. This is considerably less than results obtained in either mouse or human studies which used peritoneal or monocyte derived macrophages (Remington et al, 1972; Anderson and Remington, 1974). The reason for this is unclear, ~~but~~ there are several possibilities.

At the end of lactation, the integrity of the glandular epithelium breaks down and areas are resorbed. Considerable numbers of lymphoid cells and macrophages invade the interstitial tissues between the islands of epithelial cells which remain. The

function of the macrophages is in part to prevent bacterial opportunism at this time and also to remove cell debris and fat from the gland. When LPS is used to elicit the macrophage population, non-resident cells would be expected to be derived from outside the gland, however from a study of the literature the site does not appear to have been identified. Even taking into account the influx of some cells, it was apparent that a considerable proportion of the cells in this study contained much phagocytosed lipid, (Fig 8.4) which was readily detected with a Sudan IV stain. Thus it is possible that some or all of these macrophages were either less able or incapable of further phagocytosis.

Another possible explanation for the relatively low number of intracellular organisms found is that the macrophages obtained from the mammary gland have different properties from those derived from other sites. A previous study which used murine lung macrophages (Rying and Remington, 1977) found that these cells had a lower infection rate with Toxoplasma tachyzoites when compared with peritoneal cavity cells from the same animal. Furthermore in previous work with pseudomonas (Reynolds, Kazmierowski and Newball, 1975), human lung macrophages were found to require time to adjust to the culture conditions before optimal rates of phagocytosis occurred. As the macrophages in this study were in culture for a relatively short time, it is possible that they had not adjusted to the in vitro conditions and their phagocytic potential was reduced.

Initially, live RH strain tachyzoites were added to the macrophage monolayers, However, there was no discernible difference when the tachyzoites were incubated in the presence of immune serum or gnotobiotic serum. There was individual animal variation. This

finding does not agree with previous reports which used mouse and human macrophages where it was found that a substantial increase in the number of intracellular organisms had occurred (Remington et al, 1972; Anderson and Remington, 1974). Tachyzoites have the ability to enter cells by active penetration, therefore in the assay used in this study both the macrophage's ability to phagocytose and the tachyzoite's capability to enter cells were measured. In an attempt to separate these parameters, tachyzoites which had been inactivated with formalin were used. Over the time course examined, it was found that prior incubation of killed tachyzoites with immune serum significantly increased the proportion of cells which contained organisms and the number per cell. Macrophages exposed to tachyzoites alone or in the presence of gnotobiotic serum did eventually phagocytose some parasites.

From these findings it appears that ovine anti-toxoplasma antibody is capable of opsonising formalin inactivated tachyzoites.

The reason why specific antibody was able to opsonise formalin inactivated tachyzoites but not live organisms is unknown. Since multiplication would not have taken place during the time course of the experiment the results cannot be explained in terms of increased numbers of organisms. Furthermore it cannot be accounted for by insufficient cells since less than half the cells contained intracellular organisms. One possibility is that different populations of macrophages were obtained for each study but as, the same sheep and experimental procedures were used throughout, this seems unlikely. It is also feasible that some of the secretory products of Toxoplasma may have blocked the antibody preventing it from coating the surface of the tachyzoites or that the antibody

was internalised by the organisms. However, there is no direct evidence for this suggestion and it has not been previously reported in the literature.

From the data obtained there is no apparent difference between macrophages from Toxoplasma infected animals and those from control ewes given mouse brain. It is well established that activated macrophages are capable of enhanced phagocytosis when compared with those from normal animals (Cohn, 1978). However other studies with Toxoplasma have not been able to demonstrate large differences in the phagocytosis of tachyzoites by macrophages from either Toxoplasma infected or control animals. This may be due to the ability of the tachyzoites to actively penetrate cells and therefore mask any increase in phagocytosis.

In the system that was used here there are other possible explanations for the similarity of response of the two populations of cells. It is possible that the macrophages became activated after the ewes were infected with Toxoplasma tissue cysts but that by the time of harvesting this population was substantially reduced. To support this it is known that while in mice, macrophages activated by Toxoplasma are present for the animal's life time (Remington et al, 1972), macrophages from infected humans required in vitro stimulation with sensitised T cells before the characteristics of activation become apparent (Anderson and Remington, 1974).

It is also known that elicited macrophages, that is macrophages which have been chemically stimulated, have an increased rate of phagocytosis compared to normal macrophages. Since LPS was used to

obtain cells from both infected and uninfected sheep, it might be that both populations were chemically stimulated such that they had increased phagocytic capacity irrespective of whether they were immunologically activated.

CONCLUSIONS

No discernible difference was found in the rate of phagocytosis by mammary gland macrophages obtained from sheep inoculated with Toxoplasma or uninfected mouse brain. This may be due either to the ability of tachyzoites to actively penetrate cells or because both populations of cells were elicited with LPS. Increased phagocytosis was observed when the cells were exposed to inactivated parasites which had previously been incubated with ovine anti-toxoplasma serum although this was not demonstrated when live organisms were used.

TABLE 8.1 Inoculation of sheep with either Toxoplasma or uninfected mouse brain.

GROUP	NO. OF SHEEP	INOCULATION S.C.
1	3	100 T.gondii tissue cysts
2	3	Mouse brain

TABLE 8.2 Treatment of RH strain tachyzoites

TREATMENT	INCUBATION FOR 30 MINUTES AT 37°C
A	Tachyzoites + Immune serum
B	Tachyzoites + Gnotobiotic serum
C	Tachyzoites only

FIGURE 8.1 Mammary gland macrophages after 30 minutes incubation with tachyzoites stained with Weigert's haematoxylin.

x 1000

FIGURE 8.2 Mammary gland macrophage after 30 minutes incubation with tachyzoites showing internalised organism. The preparation was stained by Weigert's haematoxylin.

x 1000

FIGURE 8.3 Mammary gland macrophage after 60 minutes incubation with tachyzoites showing multiple infection of a cell Weigert's haematoxylin used as the stain.

x 1000

FIGURE 8.4 Mammary gland macrophage which contains large amounts of lipid. The preparation was stained with Weigert's haematoxylin.

x 1000

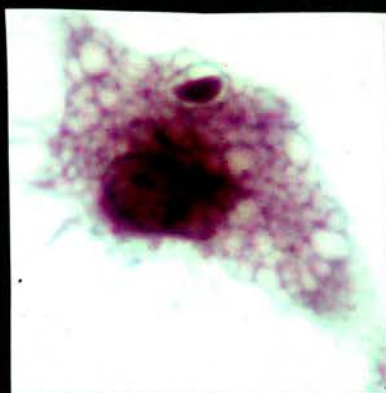
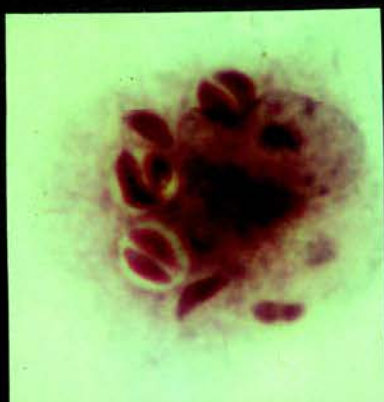
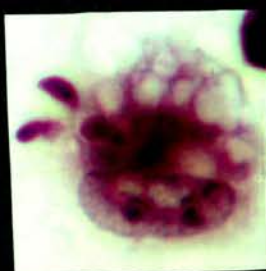
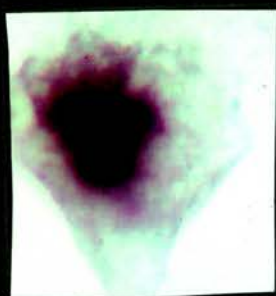


FIGURE 8.5 Per cent of cells with intracellular
tachyzoites after 30 minutes.

Treatment A - Tachyzoites + Immune serum

Treatment B Tachyzoites + Gnotobiotic serum

Treatment C Tachyzoites only

The first three columns refer to
macrophages from Toxoplasma sheep whilst
the last three are macrophages from sheep
given uninfected mouse brain.

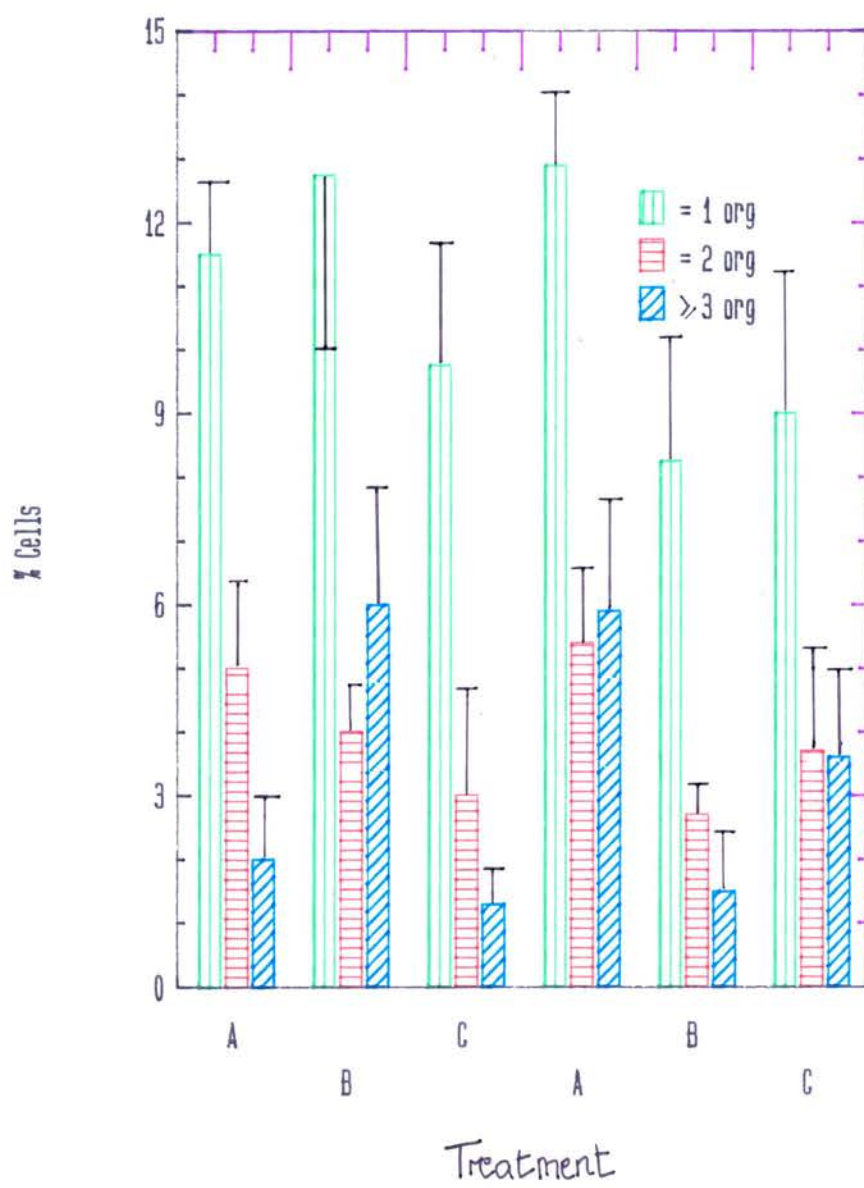


FIGURE 8.6 Per cent of cells with intracellular
tachyzoites after 60 minutes incubation.

Treatment A Tachyzoites + Immune serum

Treatment B Tachyzoites + Gnotobiotic serum

Treatment C Tachyzoites only

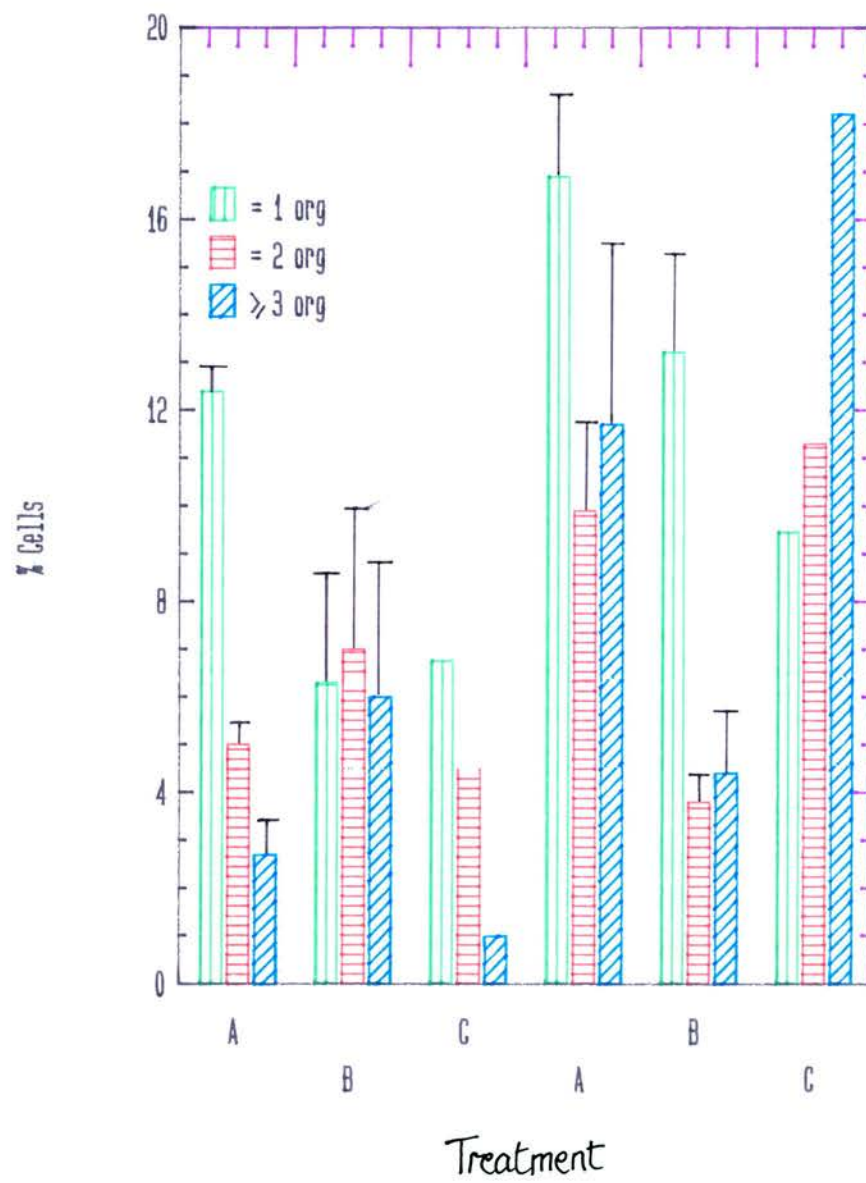


FIGURE 8.7 Per cent of cells which contained formalin
inactivated tachyzoites

Treatment A Tachyzoites + Immune serum

Treatment B Tachyzoites + Gnotobiotic serum

Treatment C Tachyzoites only

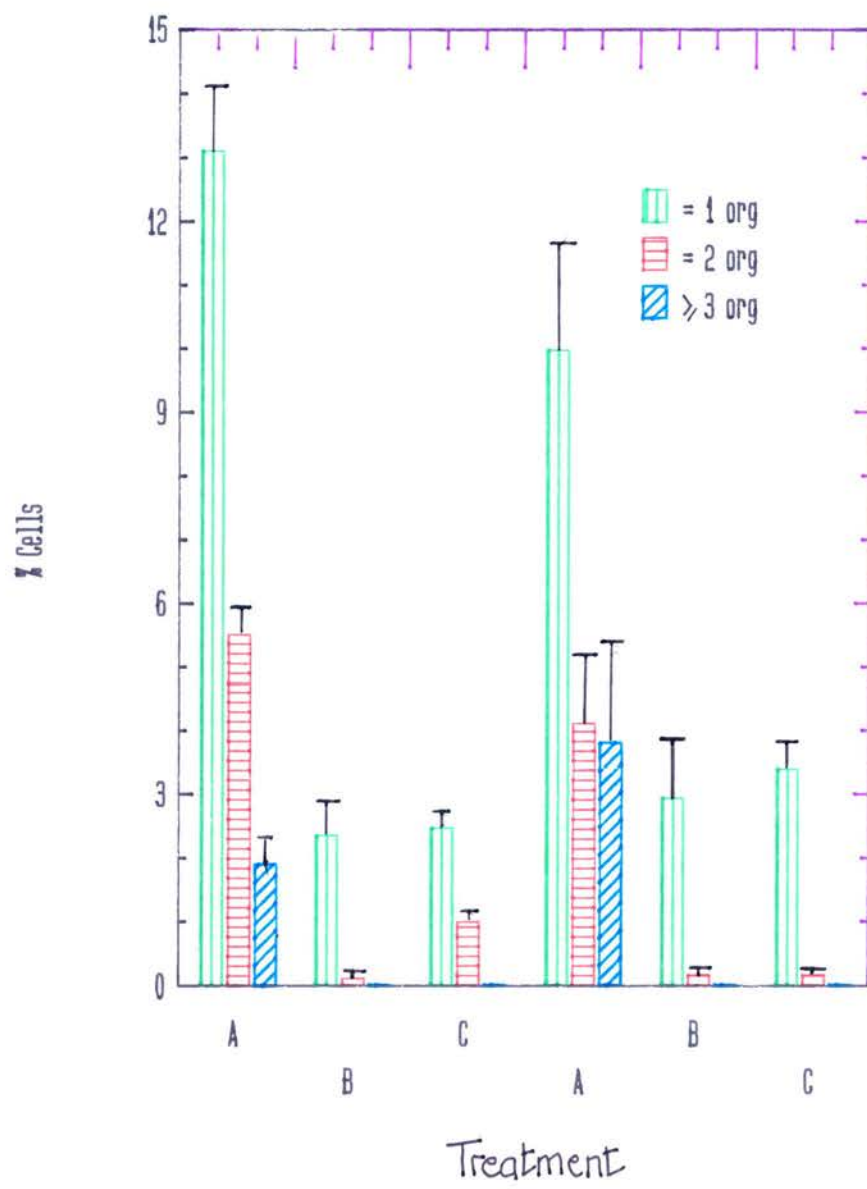
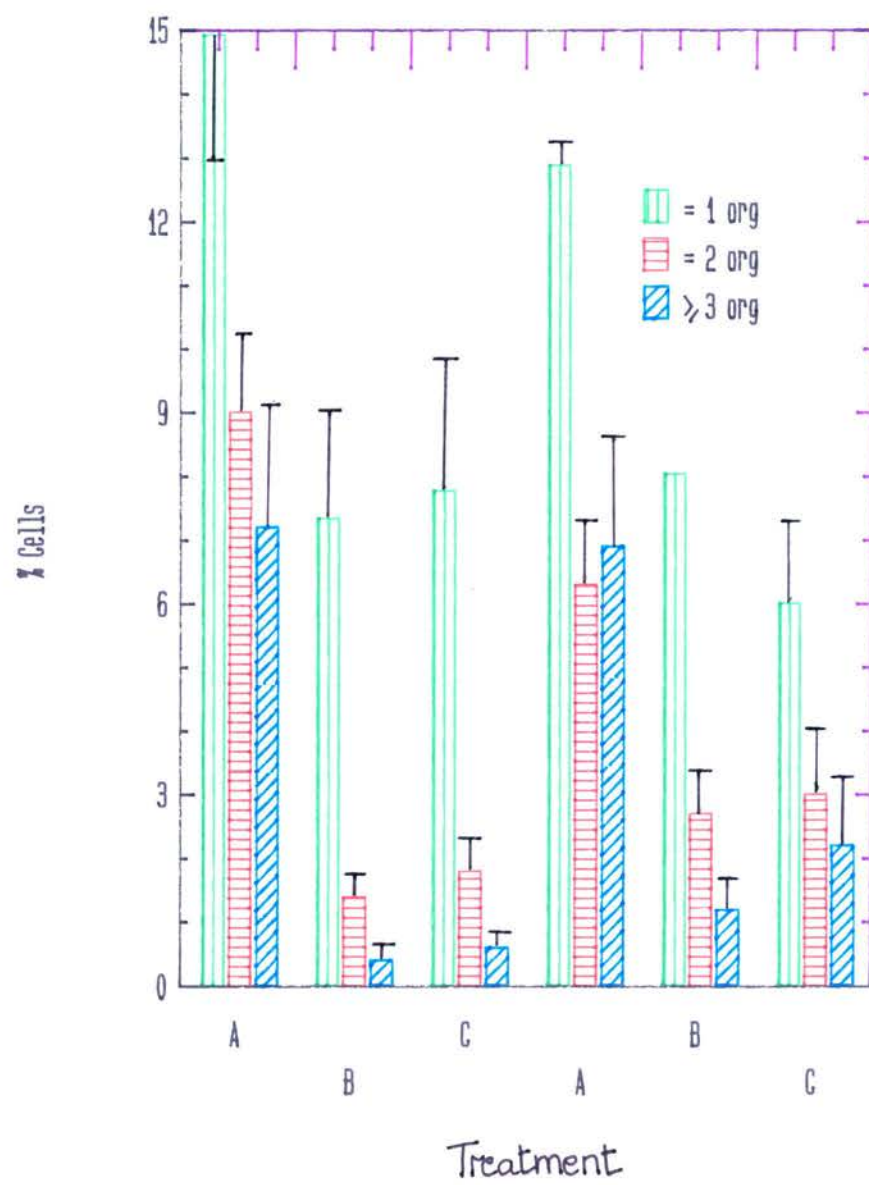


FIGURE 8.8 Per cent of cells which contained formalin
inactivated tachyzoites after 60 minutes
incubation.

Treatment A Tachyzoites + Immune serum

Treatment B Tachyzoites + Gnotobiotic serum

Treatment C Tachyzoites only



CHAPTER 9 Analysis of Toxoplasma antigens recognised by ovine
and murine sera

INTRODUCTION

The antigenic structure of T.gondii has been investigated over the last twenty years by a variety of techniques. Of these SDS-PAGE has been used extensively since it has excellent resolution and allows the simultaneous separation of several preparations. However difficulties can arise due to the fragile nature of the gel. The development of the western blotting system, where polypeptides separated by SDS-PAGE are transferred electrophoretically to a solid phase, has been invaluable. Nitrocellulose membranes are frequently used as the solid phase support and this permits the use of immunochemical methods for the analysis of polypeptides.

To enable further understanding of the host's immune response to T.gondii it is necessary to establish which parasite components are immunogenic, if any of these are stage specific and to establish which antigens actually activate the cells responsible for the induction of protective immunity. It may then be possible to use these antigens to formulate a vaccine.

One approach to this problem is to use western blotting to analyse the host's antibody response to Toxoplasma. Several workers have employed human antisera in this system (Partaen et al, 1983; Partaen et al, 1984; Sharma et al, 1983), but to date there are no reports in which sheep sera have been analysed in this way. Thus it was decided to investigate and compare the specificity of ovine and murine anti-toxoplasma antibody at various times after inoculation with oocysts.

Since ingestion of oocysts is the major source of infection in sheep, it was considered worthwhile to determine if there were any oocyst-specific antigens recognised by the sheep's immune system.

EXPERIMENTAL PROCEDURE

The sera used in the following experiments were collected from mice and sheep experimentally infected with T.gondii as described in Chapters 4, 5 and 6. The PAGE and western blotting procedures for the separation and analysis of Toxoplasma antigens were carried out as previously described (Chapter 2).

Experiment 1 Detection of Toxoplasma antigens by mouse sera.

Groups of six mice were inoculated i.p. with 500 untreated or irradiated Toxoplasma oocysts. Two mice from each group were killed nine, 19 and 28 days after inoculation and blood collected from them (Table 9.1). Serum samples were tested by western blotting.

Experiment 2 Detection of Toxoplasma antigens by sera collected from sheep orally dosed with oocysts.

Individual sheep were given 2000, 200 or 20 oocysts orally and then challenged with 10,000 oocysts 20 weeks later, as previously described (Chapter 4). Serum samples collected before and at several time points after the inoculations were tested by western blotting (Table 9.2). Gnotobiotic lamb serum obtained at MR1 was used as a control.

Experiment 3 Detection of Toxoplasma antigens by sera collected from sheep inoculated s.c. with oocysts

Individual sheep were inoculated s.c. with untreated or irradiated Toxoplasma oocysts as previously described in Chapter 6. Serum samples were collected from each animal before and four, eight, 12, 17 and 28 days after inoculation (Table 9.3) and were examined by western blotting.

Experiment 4 Preparation of solubilised Toxoplasma oocysts and analysis of their antigenic structure.

Sporulated oocysts which had been isolated from cat faeces, were solubilised and then separated by PAGE as described below.

RESULTS

Experiment 1 detection of Toxoplasma antigens by mouse sera

The solubilised preparation of RH Toxoplasma was stained with comassie blue after separation by PAGE (Fig. 9.1). No antigens were detected by sera collected nine days after inoculation by western blotting irrespective of whether untreated or irradiated oocysts were used as the inoculum. The following results are the combined data for the two mice in each group. Sera collected on day 19 from mice given 500 untreated oocysts detected two antigens (Molecular weights 23 and 31 kD), whilst a further four bands with higher molecular weights (57, 49, 46 and 35 kD) were visible when sera collected 28 days after inoculation were tested (Figs. 9.2 and 9.3). Serum samples from mice given oocysts irradiated with 5 krad detected the same antigens as described above, however the 35 kD band was visible at an earlier stage.

Sera collected 19 days after inoculation with oocysts irradiated with 10 krad detected the 23 and 31 kD antigens and the 35 kD band was visible by day 28. However serum samples collected at these time points from control mice or those given oocysts treated with 20 or 40 krad of irradiation failed to detect any Toxoplasma antigens.

Experiment 2 Detection of Toxoplasma antigens by sera collected from sheep orally dosed with oocysts.

The five serum samples collected from each of the three animals after they had been given either 2000, 200 or 20 oocysts and subsequently challenged with 10,000 oocysts all detected three antigens (23, 33 and 68 kD). However these bands were also visible, albeit more faintly, when preinoculation samples were tested, although they were not detected by the gnotobiotic serum.

Experiment 3 Detection of Toxoplasma antigens by sera collected from sheep inoculated s.c. with oocysts.

Three faint bands (molecular weights 36, 53 and 68 kD) were visible when preinoculation sera were tested whereas no bands were apparent when gnotobiotic lamb serum was used.

No other bands were visible when sera collected four days after inoculation were tested, irrespective of the dose of untreated or irradiated oocysts the sheep received.

Only one antigen (77 kD) was detected by sera collected from the sheep given 20 oocysts at eight, 12, 17 and 28 days post inoculation (Fig. 9.5).

Eight days after inoculation serum collected from the animal given 200 oocysts detected five antigens (30, 31, 40, 51 and 89 kD). No bands were visible when day 12 serum was treated but the samples obtained 17 and 28 days after inoculation detected the same five antigens as described above.

It was not until 17 days after inoculation that serum, collected from the sheep that received 10 krad irradiated oocysts, detected any antigens. A total of seven bands were visible (30, 31, 35, 60, 69, 77 and 80 kD) and the same bands were found when day 28 serum was tested.

In contrast to this, only two bands (28 and 42 kD) were apparent when serum collected 17 days after inoculation from the sheep given 2000 oocysts was tested and only the 42 kD antigen was detected by the serum sample from 28 days after inoculation.

No bands were visible when serum collected eight, 12 or 17 days after inoculation with 5 krad irradiated oocysts was tested while the day 28 serum from this animal detected a 42 kD antigen.

Experiment 4 Preparation of solubilised Toxoplasma oocysts and analysis of their antigenic structure

In order to analyse the antigenic nature of Toxoplasma oocysts a solubilised preparation was required.

A sample of sporulated oocysts was washed three times with HBSS by centrifugation at 2000g to remove any sulphuric acid, such that the pellet obtained after the third wash contained 3×10^6 oocysts. These were resuspended in 500 μ l of SDS sampling

buffer. At this stage it was noted that the sporocysts appeared granular although the structure of the oocysts remained intact. Two procedures were then carried out in an attempt to fracture the oocysts' walls. Firstly the suspension was homogenised at 4°C with a glass-teflon Tri-Ry homogeniser for four periods of five minutes and secondly it was freeze thawed three times. Microscopic examination after both of these operations revealed that the oocysts were still intact. However as the size of the sample decreased with every procedure it was considered worthwhile attempting to separate potential antigens by electrophoresis since it was possible that the permeability of the oocysts' wall had been altered and would permit outward diffusion of antigenic material from the sporozoites.

Accordingly the sample was boiled for six minutes, but after this time microscopic examination revealed that some whole oocysts were still present. Irrespective of this finding PAGE was performed as previously described (Chapter 2) except that a 15 well comb was used. Following electrophoresis it was noted that a large proportion of the preparation was retained in the stacking gel. However despite these technical difficulties two bands with molecular weights of 73 and 60 kD were visible in the separating gel (Fig. 9.7).

DISCUSSION

Western blotting was used to analyse the Toxoplasma antigens recognised by sera from experimentally infected mice and sheep. The antigen preparation used in all these experiments was derived from whole RH strain tachyzoites which had been solubilised with

SDS. After SDS-PAGE at least 20 bands were discernible by coomassie blue staining. Not all of these bands were visible when either mouse or sheep sera were used in the immunodetection procedures. However in this system the reagents used only allowed for the detection of IgG antibodies so one cannot rule out the possibility that some Toxoplasma proteins only induce an IgM response, although it seems unlikely that IgM would recognise a wider range of antigens than IgG.

Mice developed antibody against Toxoplasma antigens following inoculation with either 500 untreated or 500 5 krad irradiated oocysts. However there was some variation in the order of appearance of these antibodies although none could be detected as early as nine days after inoculation. Only the three antigens with lower molecular weights were detected by serum from mice given 10 krad irradiated oocysts. These findings support both the ELISA results and the protection experiments (Chapter 5) which indicated that the antigenic nature of the oocysts does not appear to be altered by treatment with low degrees of irradiation (5 krad).

Sera collected from mice given oocysts treated with the higher doses of irradiation (20 and 40 krad) were unable to detect any Toxoplasma antigens, which agrees with the mouse IgG-ELISA results (Chapter 5). However it should be noted that both the western blotting and the mouse IgG - ELISA used tachyzoite antigen preparations. Therefore only antibodies to sporozoite antigens which cross react with tachyzoite antigens would be detected in these tests, not oocyst-stage specific antigens. So it is possible that irradiation doses greater than 20 krad prevent sporozoites from coming into contact with the host's immune system, perhaps by

preventing excystment, and thus irradiation may not affect the antigenicity of the oocyst wall.

The data obtained from the sheep experiments is more difficult to interpret. In experiment 3 when sheep were given untreated oocysts s.c. the animals that received 200 oocysts developed antibodies to five Toxoplasma antigens by eight days after inoculation yet the sheep given 2000 oocysts recognised only two antigens (28 and 42 kD) at 17 and 28 days post injection, neither of which was detected by the former animal.

The serum from the sheep given oocysts treated with 10 krad of irradiation recognised more antigens (seven) than the animal that received 5 krad irradiated oocysts. The reason for these findings is unclear. Since the sera had all given high readings in the IgG-ELISA when tested soon after collection, it may be that technical difficulties were to blame, such as bad transfer between the gel and the nitrocellulose membrane or alternatively that repeated freeze thawing of these particular test sera had denatured some of the antibody.

The three animals in experiment 2 given either 2000, 200 or 20 oocysts orally all recognised three antigens. Although there was variation in the intensity of the staining at different time points this did not follow any specific pattern in relation to the dose of oocysts or the time after inoculation. Furthermore preinoculation serum samples from these sheep also detected these three antigens whereas gnotobiotic lamb serum did not.

Although these lines were very faint which suggests non-specific staining (and it has previously been observed that adult sheep sera gives higher levels of background staining than sera from other species, A.J. Herring, Moredun Research Institute, Personal communication), the finding that these bands increase in intensity after inoculation which Toxoplasma makes this improbable. A more likely explanation is that cross reactions exist between T.gondii and another sheep parasite. As it has previously been suggested that Toxoplasma cross-reacts with Sarcocystis (Uggla, 1986) and it is very likely that the animals in this study had previously been infected with sarcocystis, the inoculation of Toxoplasma oocysts could have boosted the antibody response to the cross-reactive antigens.

All the preinoculation sera were tested by IgG-ELISA and were found to be negative yet a similar antigen preparation was used in both tests. So it seems likely that western blotting is a more sensitive technique than ELISA.

From the results obtained it appears that antibodies to different Toxoplasma antigens are developed depending on the route of infection. However more sheep would have to be tested before this could be confirmed. It also seems that the antibody response to irradiated oocysts (10 krad) is delayed in onset in sheep but whether this is because most of the sporozoites were killed leaving only a small number of viable organisms to initiate the infection or whether irradiation has caused a defect in the multiplication rate in all the sporozoites, is not known.

The attempt to study the antigenic structure of the Toxoplasma oocyst proved to be very difficult. The only other study (Kasper et al, 1984) described the mechanical excystment of the oocysts before sonication. Due to the small number of oocysts (3×10^6) available in my study and also in the interests of safety it was decided to inactivate the intact oocyst. It is now apparent that boiling in SDS-sampling buffer is not sufficient to disintegrate the oocyst's wall. The separation by SDS-PAGE was incomplete indicating that very high molecular weight fragments were still present in the sample. Due to the lack of material it was not possible to characterise the two polypeptides which were present in the separating gel after electrophoresis.

Considerable variation existed between individual sheep and between the sheep and mouse results. However several antigens were detected by both species (23, 37 and 68 kD). All of the sheep in experiments 2 and 3 were immune to subsequent challenge with Toxoplasma oocysts, this suggests that an antibody response to particular Toxoplasma antigens may not be critical to mounting an effective immune response. The fact that far fewer antigens were detected with mouse or sheep sera than had previously been reported for human sera (Sharma et al, 1983; Partaen et al, 1983; Partaen et al, 1984) suggests that differences in the antibody response to Toxoplasma exists between species.

CONCLUSIONS

Considerable variation in the range of Toxoplasma antigens recognised by individual sheep and mouse sera was found by western blotting. However three antigens (23, 37 and 68 kD) were detected by both species.

Solubilisation of Toxoplasma oocysts was difficult but two bands (mol.wt. 60 and 73kD) were visible after PAGE.

TABLE 9.1 Procedure for Experiment 1 - Sera collected from mice inoculated i.p. with Toxoplasma oocysts were tested by western blotting. The detection probe was protein A linked to ^{125}I .

NO. OF MICE	DOSE OF OOCYSTS (IRRADIATION KRAD)	NO. OF MICE SERA COLLECTED FROM			
		DAY 0	DAY 9	DAY 19	DAY 28
6	500 (NIL)	2	2	2	2
6	500 (5)	2	2	2	2
6	500 (10)	2	2	2	2
6	500 (20)	2	2	2	2
6	500 (40)	2	2	2	2
6	NIL (-)	2	2	2	2

TABLE 9.2 Procedure for Experiment 2 - Sera collected from sheep orally dosed with Toxoplasma oocysts were tested by western blotting. The detection antiserum was Goat anti sheep IgG linked to 125 I

NO. OF SHEEP	INITIAL DOSE/ CHALLENGE DOSE OF OOCYSTS	WEEKS AFTER INOCULATION SERA COLLECTED	WEEKS AFTER CHALLENGE SERUM COLLECTED
1	20/10,000	0, 2, 6	2, 5, 7
1	200/10,000	0, 2, 6	1, 2, 5
1	2000/10,000	0, 2, 6	2, 5, 7

TABLE 9.3 Procedure for Experiment 3 - Sera collected from sheep inoculated s.c. with Toxoplasma oocysts were tested by western blotting. The detection antiserum was goat anti sheep IgG linked to 125 I.

NO. OF SHEEP	DOSE OF OOCYSTS (IRRADIATION KRAD)	DAYS AFTER INOCULATION SERA COLLECTED
1	20 (NIL)	0, 4, 8, 12, 17, 28
1	200 (NIL)	0, 4, 8, 12, 17, 28
1	2000 (NIL)	0, 4, 8, 12, 17, 28
1	2000 (5)	0, 4, 8, 12, 17, 28
1	2000 (10)	0, 4, 8, 12, 17, 28

FIGURE 9.1 T. gondii RH strain tachyzoites
solubilised in SDS sampling buffer,
separated by SDS-PAGE and subsequently
stained with coomassie blue.

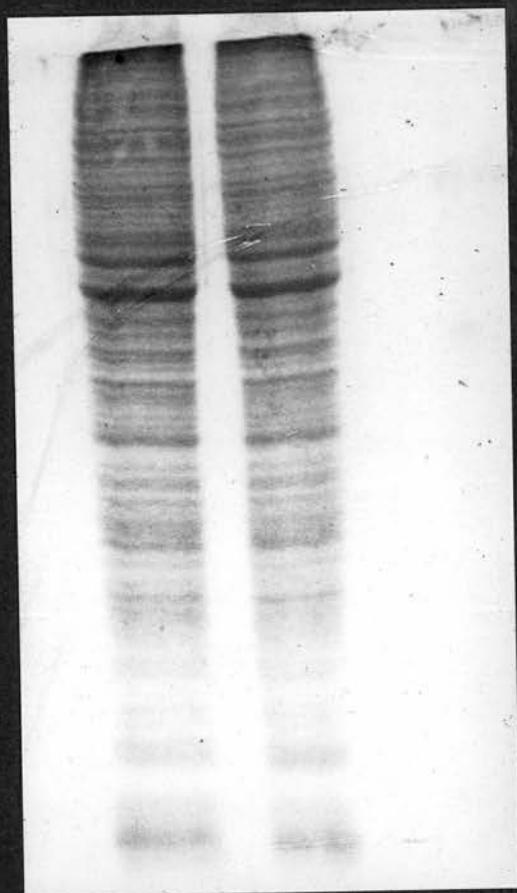


FIGURE 9.2 Autoradiography of Toxoplasma antigens
detected by mouse sera following i.p.
inoculation with oocysts.

Track	Dose of Oocysts (Irradiation krad)	Days after inoculation serum collected
1	500	28
2		19
3		9
4	500 (5)	28
5		19
6		9
7	500 (NIL)	28
8		19

FIGURE 9.3 Autoradiography of Toxoplasma antigens
detected by mouse sera following i.p.
inoculation with oocysts.

Track	Dose of oocysts (irradiation krad)	Days after inoculation serum collected
1	500 (10)	28
2		19
3		9
4	500 (5)	28
5		19
6		9
7	500 (NIL)	28
8		19
9		9

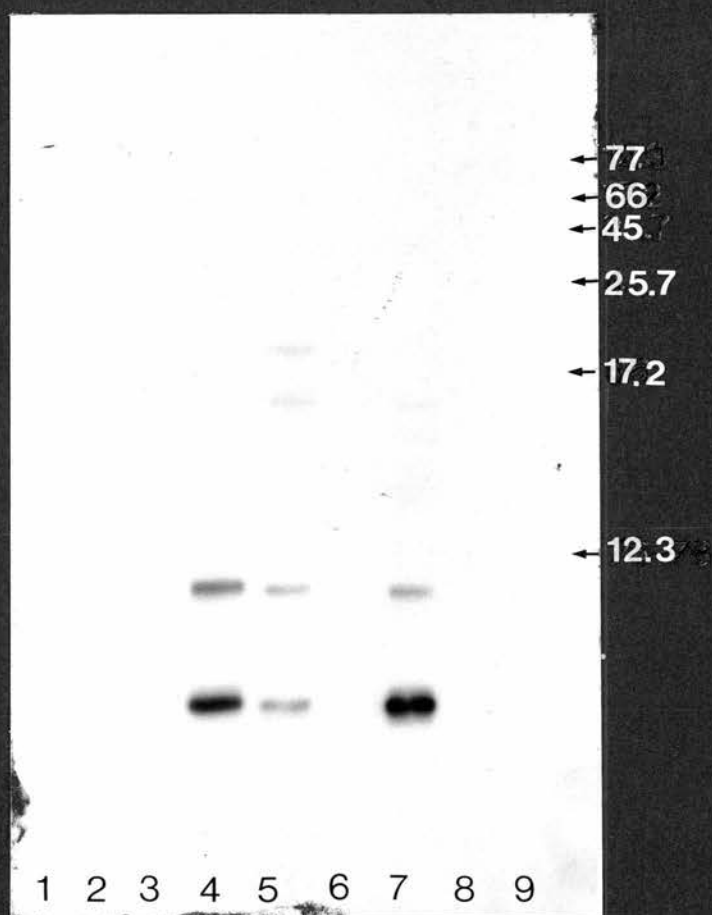
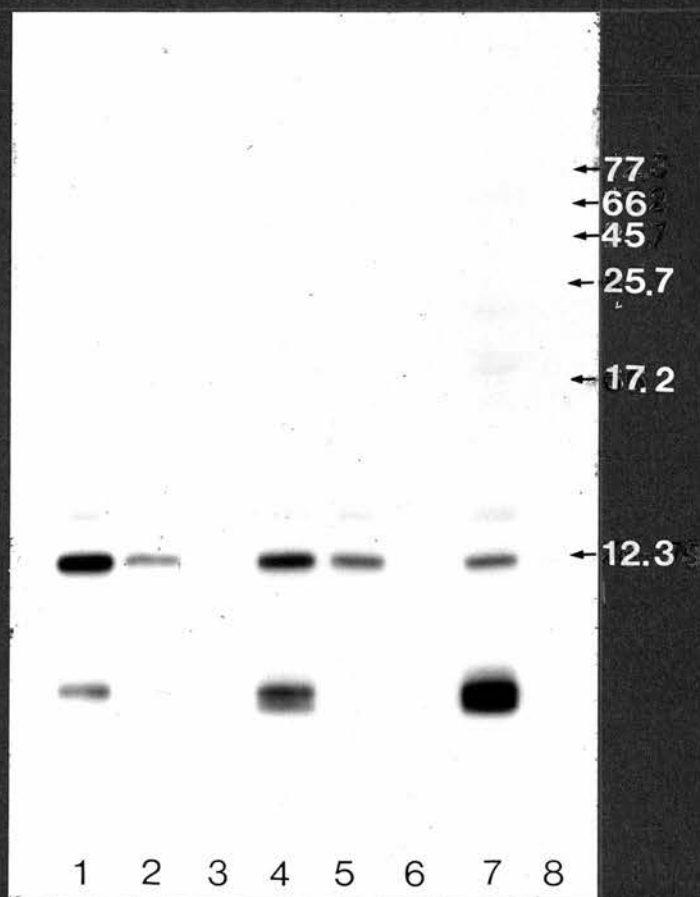


FIGURE 9.4 Autoradiograph of western blot with test sera from sheep orally dosed with oocysts.

Track	Initial Dose/ Challenge Dose of oocysts	Weeks after Initial dose serum collected	Weeks after Challenge Serum collected
1	20/10,000	0	-
2	"	2	-
3	"	6	-
4	"	-	2
5	"	-	5
6	"	-	7
7	200/10,000	0	-
8	"	2	-
9	"	6	-
10	"	-	1
11	"	-	2
12	"	-	5
13	2000/10,000	0	-
14	"	2	-
15	"	6	-
16	"	-	2
17	"	-	5
18	"	-	7

FIGURE 9.5 Autoradiograph of western blot with test sera from sheep inoculated s.c. with oocysts.

Track	Dose of oocysts (irradiation krad)	Days after Inoculation Serum collected
1	20 (NIL)	0
2	"	4
3	"	8
4	"	12
5	"	17
6	"	28
7	200 (NIL)	0
8	"	4
9	"	8
10	"	12
11	"	17
12	"	28
13	2000 (10)	0
14	"	4
15	"	8
16	"	12
17	"	17
18	"	28
19	Gnotobiotic serum	

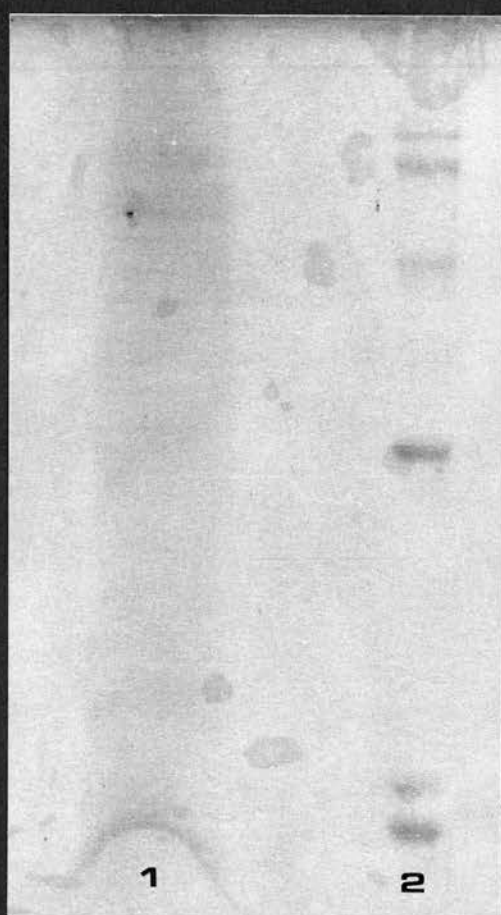
FIGURE 9.6 Autoradiograph of western blot with test sera from sheep inoculated s.c. with oocysts.

Track	Dose of oocysts (irradiation krad)	Days after Inoculation Serum collected
1	2000 (5)	12
2	"	17
3	"	28
4	2000 (NIL)	0
5	"	4
6	"	8
7	"	12
8	"	17
9	"	28

FIGURE 9.7 Coomassie blue staining of SDS-PAGE separation of oocyst preparation

Track 1 Oocyst suspension

Track 2 Molecular weight standard



CHAPTER 10 General Discussion

INTRODUCTION

The main aim of the experiments described in this thesis was to extend our understanding of the ovine immune response to T.gondii infection. Evidence obtained from studies in other species have demonstrated that both humoral and cellular responses occur after infection (Frenkel, 1967; Lindberg and Frenkel, 1977; McCabe et al, 1984; Hughes et al, 1985). I therefore attempted to examine both parameters in sheep that were experimentally infected with T.gondii, by measuring specific anti-toxoplasma antibody, monitoring the cell output from efferent lymph, examining infected lymph nodes with immunohistological techniques, measuring the phagocytic capacity of mammary gland macrophages and by determining the parasite's immunogenic components.

As individual results are discussed in the relevant chapters, in this section I will attempt to bring together my findings and discuss them in relation to the sequence of events which occur before and after a sheep becomes infected with T.gondii.

ACUTE INFECTION

Toxoplasma is a ubiquitous parasite but its sexual cycle can only take place in the intestines of susceptible cats and this leads to the production of oocysts which are excreted in the faeces (Chapter 1). While this may occur after infection with any of the forms of Toxoplasma the ingestion of bradyzoites in tissue cysts leads to the largest number of oocysts (Dubey and Frenkel, 1976) and thus most oocysts are shed after cats have eaten small animals chronically infected with the parasite.

Cats usually begin to shed oocysts four days after ingesting tissue cysts and continue to do so for a further six days. The number of parasites excreted varies but over 2×10^7 were found in one day's collection of faeces from a single cat (Chapter 3). Due to the resistant nature of the oocysts, it is likely that infected faeces can contaminate food, pasture and bedding on the farm for a considerable period of time and thus sheep can become infected by ingestion of this material (Faull, Clarkson and Winter, 1986). As ingestion of as few as 200M1 strain oocysts is sufficient to induce infection in an adult sheep (Chapter 4) then oocysts excreted from one cat could very readily be the source of infection for a substantial number of sheep.

Following ingestion by a sheep the oocysts excyst in the intestine, (it is of interest that excystment is not necessarily restricted to the intestine as infection can still occur after experimental s.c. inoculation of sporulated oocysts, Chapter 6). The sporozoites which are released penetrate the gut wall and almost certainly travel via the lymphatics to the draining mesenteric lymph nodes, and this is supported by the observations of Dubey (1984) who found Toxoplasma in these nodes in larger numbers than in other tissues four days after orally administering oocysts to sheep.

On arriving in the lymph nodes, Toxoplasma multiplies within cells and stimulates an immune response. The parasites are released into the lymph (Buxton et al, 1981) resulting in a parasitaemia approximately four to six days after inoculation.

At this time a pyrexia can be detected (Chapter 4 and 8, Miller et al, 1982). If however a very low dose of oocysts is given the onset of the fever can be delayed for four days (Chapter 6). The factors involved in the induction of this pyrexia are not known, but since neither a parasitaemia nor a fever is found following a challenge infection it seems probable that they are related. The pyrexia may be induced by factors released from the host cells. It is known that IL-1 produced by activated macrophages has pyrogenic properties (Durum, Schmidt and Oppenheim, 1985) but unless different criteria operate during a secondary response when fever does not occur it seems unlikely that this monokine could be the sole cause of the pyrexia. Alternatively the pyrogenic substance might be produced by the multiplying parasites and thus inhibition of division during a challenge infection would prevent the parasitaemia and also the fever. It is known that efferent lymph is almost completely free of Toxoplasma following a challenge infection (Buxton et al, 1981).

Whilst the parasitaemia is ongoing there are significant changes taking place in the immune system. Histological examination of lymph nodes which drained the site of an experimental infection revealed lymphadenopathy, with increased numbers of follicles in the cortex and enlarged medullary cords and sinuses, while in more severe cases a breakdown in the architecture of the node occurred (Chapter 7; Buxton et al, 1981). Cellular changes in the efferent lymph following infection were dominated by a large blast cell response which accounted for more than 50 per cent of the cell output by day 11 and although some of these cells contained either IgM or IgG, the majority of them were Ig negative and presumed to be T cells (Chapter 7).

It is known that these cells play a central role in the immune response to Toxoplasma. T cells induce a B cell response which leads to the production of specific IgG antibody as early as seven days after inoculation with oocysts (Chapter 4). The amount of detectable antibody reaches a peak five to seven weeks later and remains high for several months. This response is directed against several parasite antigens (Chapter 9). It is probable that this antibody opsonises parasites resulting in increased phagocytosis by macrophages (Chapter 8). Human and murine studies have demonstrated that T cells also secrete lymphokines which act directly on macrophages, enhancing their capacity to phagocytose and kill Toxoplasma.

CONGENITAL INFECTION

If primary infection occurs during pregnancy the ensuing parasitaemia in the ewe allows the tachyzoites to pass from the uterine blood to parasitise and multiply, initially in the maternal cells of the placentome. The resulting cellular destruction causes micro-foci of necrosis which enlarge and involve placental villi so that with time they can be recognised macroscopically as white spots in the cotyledons.

In the early development of this focal necrosis tachyzoites can infect the foetus and parasitise many tissues. The effect this has on the developing foetus is dependent on the stage of gestation. When infection occurs during mid-pregnancy the most characteristic pathological changes develop in the brain where parasite-induced foci of inflammation and necrosis develop (Chapter 4). The inflammatory response indicates that the foetus is capable of mounting a cellular immune response in addition to the humoral

response, which is evident as specific anti-toxoplasma antibody in the serum.

Leucomalacia which is not directly related to parasite multiplication may be found in the brain later in gestation and it is presumed that this is due to cotyledonary damage decreasing the oxygen supply to the foetus near term (Buxton et al, 1982). This damage may also hinder the transfer of nutrients from the ewe to the foetus with the result that infected lambs can be born underweight (Chapter 4).

CHRONIC INFECTION

Irrespective of whether or not the ewe is pregnant, Toxoplasma tachyzoites continue to invade host cells and transform into bradyzoites which, by definition, are slow multiplying forms which accumulate to produce tissue cysts. These develop predominantly in the brain and muscles and it has been suggested that infectious organisms remain in these sites for the rest of the animal's life thereby creating a reservoir^r of infection for both carnivores and omnivores.

It is well established that following natural, and most experimental, infections the host is protected from subsequent challenge (Chapters 1,4 and 6). A key question is therefore, what is the relationship between this state of immunity and the persistence of the Toxoplasma infection?

Whether the development of the immune response induces the chronic phase of the infections is unclear, evidence from several sources indicates that it plays a role in the maintenance of the tissue cyst stage. Suppression of the host's immune system by

either drugs or disease can lead to reactivation of a chronic Toxoplasma infection which can have a fatal outcome (Naot, Araujo, Stinson and Remington, 1983; Luft, Brooks, Conley, McCabe and Remington, 1984). Furthermore in vitro studies have shown that although interferon secreted from activated T cells is not required for the formation of tissue cysts, its presence helps maintain them, thereby preventing their breakdown and the subsequent spread of bradyzoites (Jones et al, 1986).

It is possible that the presence of chronic infection is required for immunity to be effective and long lasting. If this was the case then it may be that bradyzoites would occasionally burst out of the protective tissue cyst environment, resulting in the restimulation of the immune system. However, evidence from studies with "non-persisting" strains of Toxoplasma suggests that chronic infection may not be a necessity. Two months after mice were inoculated with ts4 strain parasites no evidence of persistent Toxoplasma infection could be found. Despite this, mice treated in this way were protected from a subsequent challenge with the virulent M-771 strain one year later (Waldeland and Frenkel, 1983).

The ability of Toxoplasma in this case to induce long term immunity is encouraging and indicates that the development of a vaccine is a feasible option for the control of toxoplasmosis. Unfortunately to date there has been little success in obtaining an effective vaccine.

Studies with killed preparations have not demonstrated lasting immunity (Chapter 1). Oocysts treated with γ -irradiation were able to induce more effective immunity (Chapters 5 and 6) however

it is likely that a live infection was taking place leading to a persistent infection. Even if oocysts treated in this way were found to be non-pathogenic they would be unacceptable as prophylactic^c treatment for domestic animals, not only because their avirulence could not be guaranteed in all hosts, but also because the possibility of reactivation would ~~exist~~ exist and Toxoplasma is a zoonosis.

To increase the chances of producing an effective vaccine against ovine toxoplasmosis further information about the effector cells involved in response to a live infection is required. The findings described in Chapter 7 indicate that T cells may be playing a role but functional studies are needed to confirm this. The ability of macrophages to phagocytosis Toxoplasma (Chapter 8) suggests that they may also play a part in the immune response and again further work is required.

More recent attempts at vaccine development with other pathogens have concentrated on subunits of the organism. It is therefore important to continue the work with PAGE and Western blotting techniques (Chapter 9) in order to identify the particular Toxoplasma antigens which induce the immune effector cells.

After identifying the relevant antigens it will be necessary to obtain them in large quantities. One approach to this problem is by cloning the genes which code for the individual polypeptides. This approach has already been successfully used for viruses and some workers are at present using these techniques for parasite antigens (Murray, 1983; Nussenzweig and Nussenzweig, 1983).

The advantages of this technique, which relies on the production of a cDNA library, are that the proteins are produced free from any potentially infectious material and any host cell contamination. If however the required antigens are glycoproteins this procedure is more difficult since the normal vectors used are of bacterial origins and are unable to glycosylate eukaryotic proteins.

Although these may be the antigens the effective immune response is directed against, it is probable that they would have to be presented to the immune system in a form more like their arrangement in the live organism. One possible way of achieving this is to reconstitute the Toxoplasma antigens within liposomes. These are artificially produced phospholipid bilayers and they enable isolated membrane components to be inoculated in their native non-denatured configuration (Russell and Wilhelm, 1986). Another option would be to form immunostimulating complexes (ISCOMS) with the parasite antigens in order to achieve maximum stimulation of the immune system (Morein, Sundquist, Hoglund, Dalsgaard and Osterhaus, 1984).

In conclusion, while there is still insufficient knowledge on how to manufacture an effective killed vaccine, the results described here suggest that stimulation of T cells and macrophages as well as a humoral response would be required.

REFERENCES

- Adams, F.H., Kabler, P. Cooney, M. & Adams, J.M. (1949) Diagnostic tests for toxoplasmosis. *Pediatrics*. 4, 490-497.
- Aikawa, M. Komata, Y., Asai, T. & Midorikawa, O. (1977) Transmission and Scanning Electron Microscopy of Host Cell Entry by Toxoplasma gondii. *Am. J. Path.* 87, 285-296.
- Anderson, S.E. & Remington, J.S. (1974) Effect of Normal and Activated Human Macrophages on Toxoplasma gondii. *J. exp. Med.* 139, 1154-1174.
- Barlow, R.M. (1982) Neuropathology of affected sheep. *Adv. Vet. Med.* 36, 22-39.
- Bergquist, N.R. & Waller, J. (1983) A novel immunoassay for rapid detection of human IgG antibodies to Toxoplasma gondii. *J. Immun. Methods*. 61, 339-344.
- Beverley, J.K.A. (1959) Congenital Transmission of Toxoplasmosis through Successive Generations of Mice. *Nature* 183, 1348-1349.
- Beverley, J.K.A., Archer, J.F., Watson, W.A. & Fawcett, A.R. (1971) Trial of a killed vaccine in the prevention of ovine abortion due to Toxoplasmosis. *Br. vet. J.* 127, 529-535.
- Beverley, J.K.A. & Beattie, C.P. (1952) Standardisation of the dye test for toxoplasmosis. *J. clin. Path.* 5, 350-353.
- Beverley, J.K.A., Hutchison, W.M., Allsup, T.N., Spence, J.B. & Watson, W.A. (1975) Studies on the spread of Toxoplasma gondii to sheep. *Br. vet. J.* 131, 130-135.
- Beverley, J.K.A. & Watson, W.A. (1959) Ovine Abortion due to Toxoplasmosis. *Nature*. 26, 2041.
- Beverley, J.K.A. & Watson, W.A. (1962) Further studies on Toxoplasmosis and ovine abortion in Yorkshire. *Vet. Rec.* 74, 548-552.
- Beverley, J.K.A., Watson, W.A. & Payne, J.M. (1971) The Pathology of the Placenta in Ovine Abortion due to Toxoplasmosis. *Vet. Rec.* 88, 124-128.
- Beverley, J.K.A. & Watson, W.A. (1971) Prevention of experimental and of naturally occurring ovine abortion due to Toxoplasmosis. *Vet. Rec.* 88, 39-41.
- Blewett, D.A., Bryson, C.E. & Miller, J.K. (1983) Studies of antibody titres in experimentally induced ovine toxoplasmosis. *Res. Vet. Sci.* 34, 163-166
- Blewett, D.A., Miller, J.K. and Harding, J. (1983) Simple technique for the direct isolation of Toxoplasma tissue cysts from fetal ovine brain. *Vet. Rec.* 112, 98-100.
- Blewett, D.A., Teale, A.J., Miller, J.K., Scott, G.R. & Buxton, D. (1982) Toxoplasmosis in rams possible significance of venereal transmission. *Vet. Rec.* 111, 73-75.

- Blewett, D.A. and Trees, A.J. (1987) The epidemiology of ovine toxoplasmosis with especial respect to control. *Br. Vet. J.* 143, 128-135.
- Blewett, D.A. & Watson, W.A. (1984) The epidemiology of ovine toxoplasmosis. III Observations on outbreaks of clinical toxoplasmosis in relation to possible mechanisms of transmission. *Br. Vet. J.* 140, 54-63.
- Borges, J.S. & Johnson Jr., D.W. (1975) Inhibition of Multiplication of Toxoplasma gondii by Human Monocytes exposed to T Lymphocyte Products. *J. exp. Med.* 141, 483-496.
- Buergelt, C.D. & Bonde, R.K. (1983) Toxoplasmic meningoencephalitis in a West Indian manatee. *J. Am. vet. med. Ass.* 183, 1294-1296.
- Buxton, D. (1980) Experimental Infection of Athymic Mice with Toxoplasma gondii *J. Med. Microbiol.* 13, 307-311.
- Buxton, D., Blewett, D.A., Trees, A.J., McColgan, C. and Finlayson, J. Further studies in the use of monensin in the control of experimental ovine toxoplasmosis. *J. Comp. Path.* (in press)
- Buxton, D. and Finlayson, J. (1986) Experimental infection of pregnant sheep with Toxoplasma gondii: pathological and immunological observations on the placenta and foetus. *J. Comp. Path.* 96, 319-333.
- Buxton, D., Gilmour, J.S., Angus, K.W., Blewett, D.A., & Miller, J.K. (1982) Perinatal Changes in Lambs Infected with Toxoplasma gondii. *Res. Vet. Sci.* 32, 170-176.
- Buxton, D. Miller, H.R.P., Finlayson, J. & Wallace, G.R. (1981) Toxoplasma gondii : Its effect on the Ovine Popliteal Lymph Node *J. Med. Microbiol.* 14, 435-442.
- Buxton, D. Reid, H.W., Finlayson, J. & Pow, I. (1980) Immunosuppression in Toxoplasmosis : Preliminary Studies in Mice Infected with Louping-ill Virus *J. Comp. Path.* 90, 331-338.
- Camargo, M.E., Ferreira, A.W., Mineo, J.R., Takeguti, C.K. & Nakahara, O.S. (1978) Immunoglobulin G and immunoglobulin M enzyme-linked immunosorbent ~~assays~~ defined Toxoplasmosis serological patterns. *Infect. & Immun.* 21, 55-58.
- Cesbron, J.Y., Capron, A., Orlaque, G. & Santora, F. (1985) Use of a monoclonal antibody in a double-sandwich ELISA for detection of IgM antibodies to Toxoplasma gondii major surface protein (P30) *J. Immunol. Methods.* 83, 151-158.
- Chatton, E. & Blanc, G. (1917) Notes et reflexions sur le toxoplasme et la toxoplasme du gondii (Toxoplasma gondii, Nicolle et Manceaux 1909). *Arch. Inst. Pasteur Tunis.* 10, 1-41.
- Chhabra, M.B., Mahajan, R.C. & Ganguly, K. (1979) Effects of Cobalt irradiation on Virulent Toxoplasma gondii and its use in experimental immunization. *Int. J. Radiat. Biol.* 35 433-440.

Chiappino, M.L., Nichols, B.A. & O'Connor, G.R. (1984) Scanning electron microscopy of Toxoplasma gondii; parasite torsion and host cell responses during invasion. J. Protozool. 31, 288-292.

Chordi. A., Walls, K.W. & Kagan, I.G. (1964) Analysis of Toxoplasma gondii antigens by agar diffusion methods. J. Immunol. 93, 1034-1044.

Connor, R.J. & Hallwell, R.W. (1985) Serological survey of the prevalence of Toxoplasma gondii in goats and sheep in Southern Tanzania. Ann. Trop. Med. Parasit. 79, 111-112.

Conley, F.K. & Jenkins, K.A. (1981) Immunohistological study of the anatomic relationship of Toxoplasma Antigens to the inflammatory response in the brains of mice chronically infected with Toxoplasma gondii. Infect. & Immun. 31, 1184-1192.

Crowley, J.P. (1964) Abortion and perinatal mortality in sheep associated with Toxoplasmosis. Irish. J. Agric. Res. 3, 159-164.

Denmark, J.R. & Chessum, B.S. (1978) Standardization of enzyme-linked immunosorbent assay (ELISA) and the detection of Toxoplasma antibody. Med. Lab. Sci. 35, 227-232.

Desmonts, G. Couvreur, J., Alison, F., Baudelot, J., Gerbeaux, J. & Lelong, M. (1965) Etude epidemiologique sur la toxoplasmose: de l'influence de la cuisson des viandes de boucherie sur la frequence de l'infection humaine. Rev. Fr. Etud. Clin. Biol. 10, 952-958.

Desmonts, G. & Couvreur, J. (1974) Congenital toxoplasmosis: A prospective study of 378 pregnancies. N. Eng. J. Med. 290, 1110-1116.

Desmonts, G. & Remington, J.S. (1980) Direct agglutination test for diagnosis of Toxoplasma infection: method for increasing sensitivity and specificity. J. Clin. Microbiol. 11, 562-568.

Dubey, J.P. (1976) Effect of freezing on the infectivity of Toxoplasma cysts to cats. J. Am. vet. med. Ass. 165, 534-536.

Dubey, J.P. (1977) Toxoplasma, Hammondia, Bresnoitia, Sarcocystis, and other tissue cyst-forming coccidia of man and animals, in Parasite Protozoology. Vol. III Academic Press Ed. J.P. Kreier.

Dubey, J.P. (1984) Experimental Toxoplasmosis in sheep fed Toxoplasma gondii oocysts. Int. Goat and Sheep Res. 2, 93-104.

Dubey, J.P. & Frenkel, J.K. (1972) Cyst induced toxoplasmosis in cats. J. Protozool. 19, 155-177.

Dubey, J.P. & Frenkel, J.K. (1973) Experimental Toxoplasma infection in mice with strains producing oocysts. J. Parasit. 59, 505-512.

Dubey, J.P. & Frenkel, J.K. (1976) Feline toxoplasmosis from acutely infected mice and the development of Toxoplasma cysts. J. Protozool. 23, 537-546.

- Dubey, J.P., Miller, N.L. & Frenkel, J.K. (1970) Characterisation of the new fecal form of Toxoplasma gondii. J. Parasit. 56, 447-456.
- Dubey, J.P., Miller, S., Powell, E.C. & Anderson, N.R. (1986) Epidemiologic investigations on a sheep farm with Toxoplasma gondii-induced abortions. J. Am. vet. med. Ass. 188, 2.
- Dubey, J.P. & Sharma, S.P. (1980) Parasitaemia and tissue infection in sheep fed Toxoplasma gondii oocysts. J. Parasit. 66, 111-114.
- Durfee, P.T., Ma Ch Wang, C.I. and Cross J.H. (1974) Infectivity and Pathogenicity of Toxoplasma oocysts for swine. J. Parasitol. 60, 886-887.
- Durfee, P.T., Cross, S.H., Rustam, Susanto (1976) Toxoplasmosis in man and animals in South Kalimantan (Borneo) Indonesia. Am. J. Trop. Med. Hy. 25, 42-47.
- Durum, S.K. Schmidt, J.A. Oppenheim, L.L. (1985) Interleukin 1: an immunological perspective. Annu. Rev. Immunol. 3, 263-287.
- English, L.S., Morris, B. & Adams, E.P. (1976) The synthesis of immunoglobulins by lymphoid cells in the sheep. The contribution made by the lymphoblasts to the total Immunoglobulin and Antibody Production by lymphoid cells of the popliteal node during a secondary response to Bacterial Lipopolysaccharide. Cell Immunol. 27, 274-286.
- Fahey, K.J. (1980) The binding of lectins to sheep tissues and circulating cells: peanut agglutinin a marker for presumptive T-lymphocytes. Aust. J. Exp. Biol. Med. Sci. 58, 584
- Faull, W.B., Clarkson, M.J. and Winter A.C. (1986) Toxoplasmosis in a flock of sheep: Some investigations into its source and control. Vet. Rec. 119, 491-493.
- Feldman, H.A. (1956) The relationship of Toxoplasma antibody activator to the serum properdin system. Ann. N.Y. Acad. Sci. 66, 263-267.
- Feldman, H.A. & Lamb, G.A. (1966) A micromodification of the Toxoplasma dye test. J. Parasit. 52, 415.
- Ferguson, D.J.P., Hutchison, W.M. & Siim, J.C. (1975) The ultrastructural development of the macrogamete and formation of the oocyst wall of Toxoplasma gondii. Acta. pathol. microbiol. scand. 83B, 491-505.
- Filice, G.A., Yeager, A.S. & Remington, J.S. (1980) Diagnostic significance of Immunoglobulin M Antibodies to Toxoplasma gondii detected after separation of Immunoglobulin M from Immunoglobulin G Antibodies. J. Clin. Microbiol. 12, 336-342.

- Finlayson, J. (1980) A microtitre radio-immunoassay for Toxoplasma gondii antibody. J. Comp. Path. 90, 491-493.
- Fitzgerald, P.R. (1968) Effects of ionizing radiation from Cobalt 60 on oocysts of Eimeria bovis. J. Parasit. 54, 233-240.
- Fleck, D.G. (1963) Antibodies of Toxoplasma gondii. J. Hyg. (Camb.) 61, 53-60.
- Fletcher, S. (1965) Indirect fluorescent antibody technique in the serology of Toxoplasma gondii. J. Clin. Path. 18, 193-199.
- Foster, B.G. & McCulloch, W.F. (1968) Studies of Active and Passive Immunity in Animals inoculated with Toxoplasma gondii. Can. J. Microbiol. 14, 103-110.
- Franco, E.L., Sulzer, A.J., Higby, R.W. & Peralta, J.M. (1980) Immunoglobulin G and Immunoglobulin M Polar staining of Toxoplasma gondii in the indirect immunofluorescence test. J. Clin. Microbiol. 12, 780-784.
- Frenkel, J.K. (1948) Dermal hypersensitivity to Toxoplasma antigens (toxoplasmins). Proc. Soc. exp. Biol. med. 68, 634-639.
- Frenkel, J.K. (1967) Adoptive immunity to intracellular infection. J. Immunol. 98, 1309-1319.
- Frenkel, J.K. (1973) Toxoplasmosis: Parasite life cycle, pathology and immunology in "The Coccidia" Edited by D.M. Hammond and P.L. Long. University Park Press, Baltimore, Maryland. 343-410.
- Frenkel, J.K., Dubey, J.P. & Miller, N.L. (1969) Toxoplasma gondii fecal forms separated from eggs of the nematode Toxocara cati. Science. 164, 432-433.
- Frenkel, J.K. Dubey, J.P. & Miller, N.L. (1970) Toxoplasma gondii fecal stages identified as coccidian oocysts. Science. 167, 893-896.
- Frenkel, J.K. & Dubey, J.P. (1972) Rodents as vectors for Feline Coccidia Ispora Felis rivolta. J. Infect. Dis. 125, 69-72.
- Frenkel, J.K., Ruiz, A. & Chinchilla, M. (1975) Soil survival of Toxoplasma oocysts in Kansas and Costa Rica. Am. J. Trop. Med. Hyg. 24, 439-443.
- Frenkel, J.K. & Taylor D.W. (1982) Toxoplasmosis in IgM suppressed mice. Infect. & Immunol. 360-367.
- Fulton, J.D. (1965) Microagglutination test for Toxoplasma antibodies. Immunology. 9, 491-495.
- Fulton, J.D. & Fulton, F. (1965) Complement-fixation tests in Toxoplasmosis with purified antigen. Nature. 205, 776-778.
- Fulton, J.D. & Turk, J.L. (1959) Direct agglutination test for Toxoplasma gondii. Lancet ii, 1068-1069.

Gardiner, A.C., Nettleton, P.F. & Barlow, R.M. (1983) Immunology of a spontaneous and experimental mucosal disease-like syndrome in sheep recovered from clinical Border Disease. *J. Comp. Path.* 93, 463-469.

Gavin, M.A., Wanko, T. & Jacobs, L. (1962) Electron microscope studies of reproducing and interkinetic Toxoplasma. *J. Protozool.* 9, 222-234.

Gibson, C.L. and Coleman, N. (1958) The prevalence of Toxoplasma antibodies in Guatemala and Costa Rica. *Am. J. Trop. Med Hyg.* 7, 334-338.

Goldman, M. (1957) Staining Toxoplasma gondii with fluorescein labelled antibody: A new serologic test for antibodies to Toxoplasma based upon inhibition specific staining. *J. Exp. Med.* 105, 557-573.

Gordon, M.A., Duncan, R.A. & Kingsley, L.C. (1981) Automated immunofluorescence test for Toxoplasmosis. *J. Clin. Microbiol.* 13, 283-285.

Hall, J.G. and Morris, B. (1962) The output of cells in lymph from the popliteal node of sheep. *Q.J. Exp. Physiol.* 47, 360-369.

Hall, J.G., Morris, B., Moreno, G.D. & Bessis, M.C. (1967) The ultrastructure and function of the cells in lymph following antigenic stimulation. *J. exp. Med.* 125, 91-109.

Hall, J.G. & Smith, J.B. (1971) Studies on afferent and efferent lymph of lymphnodes draining the site of application of Fluorodinitro benzene (FDNB). *Immunology.* 21, 69-79.

Handman, E., Chester, P.M. & Remington, J.S. (1980) Delayed Hypersensitivity to Toxoplasma and Unrelated Antigens in Toxoplasma Infected Mice: Induction and Elicitation of DTH by Antigen Pulsed Macrophages. *Infect. Immun.* 28, 524-531.

Handman, E., Goding, J.W. & Remington, J.S. (1980) Detection and characterisation of membrane antigens of Toxoplasma gondii. *J. Immunol.* 124, 2578-2583.

Handman, E. & Remington, J.S. (1980) Antibody Responses to Toxoplasma Antigens in Mice Infected with Strains of Different Virulence, *Infect. Immun.* 29, 215-220.

Handman, E. & Remington, J.S. (1980) Serological and immunochemical characterization of monoclonal antibodies to Toxoplasma gondii. *Immunology.* 40, 579-588.

Hartley, W.J. (1961) Experimental transmission of Toxoplasmosis in sheep. *N.Z. vet. J.* 9, 1-6.

Hartley, W.J. (1964) Experimental Transmission of Toxoplasmosis in Ewes Showing High and Low Dye Test Titres. *N.Z. vet. J.* 12, 6-8.

Hartley, W.J., Jebson, J.L. & McFarlane, D. (1954) New Zealand Type II Abortion in Ewes, Aust. vet. J. 216-218.

Hartley, W.J. & Karter, J.C. (1963) The pathology of Toxoplasma infection in the pregnant ewe. Res. vet. Sci. 4, 326-332.

Hartley, W.J. & Marshall, S.C. (1957) Toxoplasmosis as a Cause of Ovine Perinatal Mortality. N.Z. vet. J. 5, 119-124.

Hartley, W.J. & Moyle, G.G. (1974) Further Observations on the Epidemiology of Ovine Toxoplasma Infection, Aust. J. exp. Bio. med. Sci. 52, 647-653.

Hauser, W.E. & Remington, J.S. (1981) Effect of Monoclonal antibodies on phagocytosis and killing of Toxoplasma gondii by normal macrophages. Infect. & Immun. 32, 637-40.

Hauser, W.E. & Tsai, V. (1986) Acute Toxoplasma infection of mice induces spleen NK cells that are cytotoxic for Toxoplasma gondii in vitro. J. Immunol. 136, 313-319.

Heath, T.J., Lascelles, A.K. & Morris, B. (1962) The cells of sheep lymph. J. Anat. Lond. 96, 397-408.

Hein, H.E. (1963) Vaccination against infection with Eimeria tenella in broiler chickens. Proc. 17th World Veterinary Congress, Hanover 2, 1443-1452.

Huffman, E.M., Kirk, J.H., Winward, L. and Gorham, J.R. (1981) Relationship of neonatal mortality in lambs to serological status of the ewe for Toxoplasma gondii. JAVMA 178, 679-682.

Hughes, H.P.A. & Balfour, A.H. (1981) An investigation of the antigenic structure of Toxoplasma gondii. Parasite Immunol. 3, 235-248.

Hughes, H.P.A., Gonzalez, A., Guhi, F. & Hudson, L. (1985) Antigenic-specific lymphocyte transformation in congenital Toxoplasmosis. Immunology Letters 10, 95-98.

Hughes, H.P.A., van Knapen, F., Atkinson, H.J. Balfour, A.H. & Lee, D.L. (1982) A new soluble antigen preparation of Toxoplasma gondii and its use in serological diagnosis. Clin. exp. Immun. 49, 239-246.

Huldt, G. (1966) Experimental toxoplasmosis. Acta Path Microbiol. Scand. 68, 605-621.

Huldt, G., Gard, S. & Olovson, S.G. (1973) Effect of Toxoplasma gondii on the Thymus, Nature. 244, 301-303.

Hulland, T.J. & Tobe, S.B. (1961) Toxoplasmosis as a cause of abortion in Ontario sheep. Can. Vet. J. 2, 45-51.

^{W.M.} Hunter and ^{F.C.} Greenwood (1962) Preparation of iodine - 131 labelled human growth hormone of high specific activity. Nature. 194, 495-496.

Hutchison, W.M. (1965) Experimental Transmission of Toxoplasma gondii. Nature. 29, 961-962.

- Hutchison, W.M. (1967) The nematode transmission of Toxoplasma gondii. Trans. R. Soc. Trop. med. Hyg. 61, 80-89.
- Hutchison, W.M., Dunachie, J.F., Siim, J.C. & Work, K. (1969) Life cycle of Toxoplasma gondii. Br. Med. J. 1, 806.
- Hutchison, W.M., Dunachie, J.F., Siim, J.C. & Work, K. (1970) Coccidian-like nature of Toxoplasma gondii. Br. Med. J. 4, 142-144.
- Igarashi, I., Taguchi, M. & Suzuki, N. (1979) Fundamental Studies on Macrophage Migration Inhibitory Factor(s) in the Supernatant from Spleen Cells in Mice Infected with Toxoplasma gondii Zbl. Bakt. Hyg., I. Abt. Orig. 244, 374-382.
- Jacobs, L. & Hartley, W.J. (1964) Ovine Toxoplasmosis: Studies on Parasitaemia, Tissue Infection and Congenital Transmission in Ewes Infected by Various Routes. Br. vet. J. 120, 347-364.
- Jacobs, L., Remington, J.S. & Melton, M.L. (1960) The resistance of the encysted form of Toxoplasma gondii. J. Parasit. 46, 11-21.
- Jarrett, W.F.H., Jennings, F.W., Martin, B., McIntyre, W.I.M., Mulligan, W., Sharp, N.C.C. and Urquhart, G.M. (1958) A field trial of a parasitic Bronchitis Vaccine. Vet. Rec. 70, 451-454.
- Jarrett, W.H.W., Jennings, F.W., McIntyre, W.I.M., Mulligan, W., Sharp, N.C.C. (1961) A pasture trial using 2 immunizing doses of a parasitic bronchitis vaccine. Amer. J. Vet. Res. 22, 492-495.
- Jennis, F. (1966) A simple haemagglutination test for Toxoplasmosis using pyruric aldehyde treated cells. Aust. J. exp. Biol. med. Sci. 44, 317-322.
- Jewell, M.L., Frenkel, J.K., Johnson, K.M., Reed, V. & Ruiz, A. (1972) Development of Toxoplasma oocysts in Neotropical felidae. Am. J. trop. Med. and Hyg. 21, 512-517.
- Johnson, A. M., McDonald, P.J. and Neoh, S.H. (1981) Molecular weight analysis of the major polypeptides and glycopeptides of Toxoplasma gondii. Biochem. Biophys. Res. Comm. 100, 934-943
- Johnson, A.M., Haynes, W.D. Leppard, P.J., McDonald, P.J. & Neoh, S.H. (1983) Ultrastructural and biochemical studies on the immunohistochemistry of Toxoplasma gondii using monoclonal antibodies. Histochemistry. 77, 209-215.
- Johnson, A.M., McDonald, P.J. & Neoh, S.H. (1983) Monoclonal antibodies to Toxoplasma cell membrane surface antigens protect mice from Toxoplasmosis. J. Protozool. 30, 351-356.
- Johnson, A.M., Roberts, H. & McDonald, P.J. (1980) Age, sex distribution of Toxoplasma antibody in the South Australian population. J. Hyg. Camb. 84, 315-320.
- Jones, T.C., Bienz, K.A. and Erb, P. (1986) In vitro cultivation of Toxoplasma gondii cysts in astrocytes in the presence of gamma interferon. Infect. Immun. 51, 147-156.

- Jones, T.C., Len, L. & Hirsch, J.G. (1975) Assessment in vitro of Immunity against Toxoplasma gondii. J. exp. Med. 141, 466-482.
- Jones, T.C., Yeh, S. & Hirsch, J.G. (1972) The interaction between Toxoplasma gondii and mammalian cells 1. Mechanism of entry and intracellular fate of the parasite. J. exp. Med. 136, 1157-1172.
- Kasper, L.H., Bradley, M.S. & Pfefferkorn, E.R. (1984) Identification of stage-specific sporozoite antigens of Toxoplasma gondii by monoclonal antibodies. J. Immun. 132, 443-449.
- Kasper, L.H., Crabb, J.H. & Pfefferkorn, E.R. (1983) Purification of a major membrane protein of Toxoplasma gondii by immunoabsorption with a monoclonal antibody. J. Immunol. 130, 2407-2412.
- Kasper, L.H. and Ware, P.L. (1985) Recognition and characterisation of Stage specific oocyst/sporozoite ags of Toxoplasma gondii antisera. J. Clin. Invest. 75, 1570-1577.
- Kean, B.H., Kimball, A.C. & Christenson, W.N. (1969) An epidemic of acute toxoplasmosis. J. Am. Med. Ass. 208, 1002-1004.
- Kline, J., Stein, Z., Susser, M. and Warburton, D. (1985) Fever during pregnancy and spontaneous abortion. Am. J. Epidemiol. 121, 832-842.
- van Knapen, F., Franchmont, J.H. & van der Lugt, G. (1982) Prevalence of antibodies to Toxoplasma in farm animals in the Netherlands, and its complication for meat inspection. Vet. Quart. 4, 101-105.
- Koestner, A. & Cole, C.R. (1961) Neuropathology of Ovine and Bovine Toxoplasmosis. Am. J. vet. Res. 22, 53-66.
- Krahenbuhl, J.L., Ruskin, J & Remington, J.S. (1972) The use of killed vaccines in immunisation against an intracellular parasite. J. Immunol. 108, 425-431.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680.
- Linklater, K.A. and Dyson, D.A. (1979) Field studies on enzootic abortion of ewes in Southeast Scotland. Vet. Rec. 105, 387-389.
- Lindberg, R.E. and Frenkel, J.K. (1977) Toxoplasmosis in nude mice. J. Parasit. 63, 219-221.
- Lovelace, J.K., Moraes, M.A.P. & Hagerby, E. (1978) Toxoplasmosis among the Ticuna indians in the state of Amazonas, Brazil. Trop. Geog. Med. 30, 295-300.
- Luft, B.J., Kansas, G., Engleman, E.G. Remington, J.S. (1984) Functional and Quantitative alterations in T lymphocyte populations in acute toxoplasmosis. J. Infect. Dis. 150, 761-767.

- Lunde, M.N. & Jacobs, L. (1967) Evaluation of a latex test for Toxoplasmosis. *J. Parasit.* 53, 933-936.
- Lunde, M.N. & Jacobs, L. (1967) Differences in Toxoplasma dye test and hemagglutination antibodies shown by antigen fractionation. *Am. J. Trop. Med. Hyg.* 16, 26-30.
- Lunde, M.N. & Jacobs, L. (1983) Antigenic differences between endozoites and cystozoites of Toxoplasma gondii. *J. Parasit.* 69, 806-808.
- Lycke, E., Carlberg, K. and Norrby, R. (1975) Interactions between Toxoplasma gondii and its host cells: function of the penetration-enhancing factor of Toxoplasma. *Infect. Immun.* 11, 853-861.
- Masihi, K.N. & Werner, H. (1978) The effect of passively transferred heterologous serum on Toxoplasma gondii in NMRI mice Influence of the treatment on course of infection and cyst formation. *Zbl. Bakt. Hyg. 1 Abt. Omg. A* 240, 135-142.
- McCabe, R.E., Luft, B.J. & Remington, J.S. (1984) Effect of murine gamma interferon on murine toxoplasmosis. *J. Infect. Dis.* 150, 961-962.
- McCohn, A.A. Hutchison, W.M. and Sim, M. (1981) The prevalence of Toxoplasma gondii in meat animals and cats in Central Scotland. *Annals of Tropical Medicine and Parasitology.* 75 157-164.
- McDowell, G.H., Lee, C.S. and Lascells, A.K. (1969) Collection of polymorphs and macrophages from the involuted mammary gland of the ewe. *Res. Vet. Sci.* 10, 13-17.
- McLeod, R., Van Le, L. & Remington, J.S. (1982) Toxoplasma gondii: Lymphocyte Function during Acute Infection in Mice. *Expl. Parasit.* 54, 55-63.
- Michel, R., Schupp, K., Raether, W. and Bierther, F.W. (1980) Formation of a close junction during invasion of erythrocytes by Toxoplasma gondii in vitro. *Int. J. Parasitol.* 10, 309-313.
- Miller, J.K., Blewett, D.A. & Buxton, D. (1982) Clinical and serological response of pregnant gimmers to experimentally induced toxoplasmosis. *Vet. Rec.* 111, 124-126.
- Miller, N.L., Frenkel, J.K. & Dubey, J.P. (1972) Oral infections with Toxoplasma cysts and oocysts in felines, other mammals and in birds. *J. Parasit.* 58, 928-937.
- Mitchell, R. G. and Green, C.A. (1960) The haemagglutination test for Toxoplasma antibodies. *J. Clin. Path.* 13, 331-335.
- Mineo, J.R., Camargo, M.E. & Ferreira, A.W. (1980) Enzyme-linked immunosorbent assay for antibodies to Toxoplasma gondii polysaccharides in human Toxoplasma. *Infect. & Immun.* 27, 283-287.

Morein, B. Sundquist, B. Hogland, S., Dalsgaard, K. and Osterhaus, A. (1984) Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature*. 308, 457-460.

Moreno, T., Martinez-Gomez, F. & Hernandez-Rodriguez, S. (1985) Toxoplasmosis in pigs in Cordoba, Spain. *Ann. Trop. Med. Parasit.* 79, 271-273.

Munday, B.L. (1970) The epidemiology of toxoplasmosis with particular reference to the Tasmanian environment of Melbourne. M.V. Sc. Dissertation.

Munday, B.L. (1972) Transmission of Toxoplasma infection from chronically infected ewes to their lambs. *Br. vet. J.* 128, lxxi.

Murphy, M.J., Hay, J.B., Morris, B. and Bessis, M.C. Ultrastructural Analysis of Antibody Synthesis in Cells from Lymph and Lymph Nodes. *Amer. J. Path.* 66, 25-42.

Murray, H.W., Byrne, G.I., Rothermel D. & Cartelli, C.D. (1983) Lymphokine Enhances Oxygen-Independent Activity Against Intracellular Pathogens. *J. exp. Med.* 158, 234-239.

Murray, H.W. & Cohn, Z.A. (1979) Macrophage oxygen-dependent antimicrobial activity I - Susceptibility of Toxoplasma gondii to oxygen intermediates. *J. exp. Med.* 150, 938-949.

Murray, H.W. & Cohn, Z.A. (1980) Macrophage oxygen-dependent antimicrobial activity III Enhanced oxidative metabolism as an expression of macrophage activation. *J. exp. Med.* 152, 1596-1609.

Murray, H.W., Rubin, B.Y., Carriero, S.M., Harns, A.M. & Jaffee, E.K. (1981) Human mononuclear phagocyte antiprotozoal mechanism: oxygen dependent oxygen independent activity against intracellular Toxoplasma gondii. *J. Immunol.* 134, 1982-1988.

Murray, K. (1983), Polypeptide production by recombinant DNA techniques, in, *New Approaches to Vaccine Development*, edited by Bell, R. and Torrigiani, G. WHO, Geneva, Switzerland.

Nagasawa, H., Igarashi, I., Matsumoto, T., Sakura, H., Marbella, C. & Suzuki, N. (1980) Mouse spleen cell-derived Toxoplasma growth inhibitory factor: its separation from macrophage migration inhibitory factor. *Immunobiol.* 157, 307-319.

Nakayama, I. (1969) Persistence of positive haemagglutination titre in animals vaccinated with killed Toxoplasmas and their resistance to challenge with virulent strain. *Jap. J. Parasitol.* 18, 91-101.

Naot, Y., Barnett, E.V. & Remington, J.S.. (1981) Method of avoiding false positive results occurring in Immunoglobulin M enzyme-linked immunosorbent assays due to presence of both rheumatoid factor and antinuclear antibodies. *J. Clin. Microbiol.* 14, 73-78.

Naot, Y., Desmonts, G. & Remington, J.S. (1981) IgM enzyme-linked immunoabsorbent assay test for the diagnosis of congenital Toxoplasma infection. *J. Pediat.* 98, 32-36.

- Naot, Y., Guptill, D.R., Mullenax, J. and Remington, J.S. (1983) Characterisation of Toxoplasma gondii antigens that react with human Immunoglobulin M and Immunoglobulin G antibodies. *Infect. Immun* 41, 331-338
- Naot, Y., Guptill, D.R. & Remington, J.S. (1982) Duration of IgM antibodies to Toxoplasma gondii after acute acquired toxoplasmosis. *J. Infect. Dis.* 145, 770.
- Naot, Y. & Remington, J.S. (1980) An enzyme linked immunosorbent assay for detection of IgM antibodies to Toxoplasma gondii: Use for diagnosis of acute acquired toxoplasmosis. *J. Infect. Dis.* 142, 757-766.
- Neu, H.C. (1967) Toxoplasmosis transmitted at autopsy. *J. Am. Med. Ass.* 202, 844-845.
- Nicolle, C. & Manceaux, L. (1908) Sur une infection a corps de Leishman (ou organisms voisins) du gondii. *C.R. Hebd. Seances Acad. Sci.* 147, 763-766.
- Norrby, R. and Lycke, E. (1967) Factors enhancing the host cell penetration of Toxoplasma gondii. *J. Bacteriol.* 93, 53-58.
- Nussenzweig, V. and Nussenzweig, R.S. (1983) Recent advances in the development of a sporozoite vaccine for malaria, in, *New Approaches to Vaccine Development*, Edited by Bell, R. and Torrigiani, G. WHO, Geneva, Switzerland.
- Olafson, P. and Monlux, W.S. (1942) Toxoplasma Infection in Animals. *Cornell Veterinarian.* 32, 176-190
- Ordonez, G.A., Newman, J.T. & Stone, M.J. (1982) Serological diagnosis of Toxoplasma gondii infections by rapid separation of serum immunoglobulins M and G with CM Bio-Gel A. *J. Clin. Microbiol.* 16, 751-753.
- Osborne, H.G. (1959) Abortion in sheep associated with Toxoplasma. *Aust. vet. J.* 35, 424-425.
- Osuyemi, T.I.O., Synge, E.M.M., Agbonlahor, D.E. & Agbavwe, R. (1985) Prevalence of Toxoplasma gondii antibody in man in Plateau State and meat animals in Nigeria. *Trans. R. Soc. trop. Med. Hyg.* 79, 21-23.
- Outteridge, P.M. Fahey, K.J. and Lee, C.S. (1981) Lymphocyte surface markers in sheep blood and lymph. *Aust. J. Exp. Biol. Med. Sci.* 59, 143-145.
- Overdulve, J.P. (1970) The identity of Toxoplasma Nicolle and Manceaux, 1909 with Isospora Schneider, 1881. *Proc. Kon. Ned. Akad. Wetensch Ser. C* 73, 129-151.
- Panigrahi, H., Mithal, S., Motiapatra, L.N. & Shyam Sundun, K. (1978) Indirect haemagglutination test and indirect fluorescent antibody tests in suspected cases of Toxoplasmosis. *Indian J. Med. Res.* 67, 918-923.

Partanen, P., Turunen, H.J., Passivuo, R., Forsblom, E., Suni, J. & Lenikki, P.O. (1983) Identification of antigenic components of Toxoplasma gondii by an immunoblotting technique. *Fems Letters* 158, 252-254.

Partanen, P., Turunen, H.J. Passivuo, R.T.A. & Lenikki, P.O. (1984) Immunoblot analysis of Toxoplasma gondii antigens by Human immunoglobulins G, M and A antibodies at different stages of infection. *J. clin. Microbiol.* 20, 133-135.

Perry, B.D. Mogollon, J.D., Grieve, A.S. & de Galvis, A.C.H. (1979) Serological study of ovine toxoplasmosis in Colombia: Epidemiological study of a field outbreak. *Vet. Rec.* 104, 231-234.

Pfefferkorn, E.R. (1984) Interferon gamma blocks growth of Toxoplasma gondii in human fibroblasts by inducing host cells to degrade tryptophan. *Proc. Natn. Acad. Sci. USA.* 81, 908-912.

Pfefferkorn, E.R. & Guyre, P.M. (1984) Inhibition of growth of Toxoplasma gondii in cultured fibroblasts by human recombinant gamma interferon. *Infect. & Immun.* 44, 211-216.

Pfefferkon, E.R. and Pfefferkon, L.C. (1976) Toxoplasma gondii: Isolation and preliminary characterisation of temperature sensitive mutants. *Exp. Parasitol.* 39, 365-376.

Philips, R.S. (1971) Immunity of rats and mice following injection with 60 Co irradiated Babesia rodhaini infected red cells. *Parasitology.* 62, 221-231.

Pinkerton, H. & Weinman, D. (1940) Toxoplasma infection in man. *Archs. Path.* 30, 374-392.

Plant, J.W., Richardson, N and Moyle, G.G. (1974) Toxoplasma infection and abortion in sheep associated with feeding of grain contaminated with cat faeces. *Aust. Vet. J.* 50, 19-21.

Price, J.H. (1969) Toxoplasma infection in an urban community. 4, 141-143.

Pyndiah, N., Krech, U., Price, P. & Wilhelm, J. (1979) Simplified chromatographic separation of Immunoglobulin M from G and its application to Toxoplasma indirect immunofluorescence. *J. Clin. Microbiol.* 9, 170-174.

Rawal, B.D. (1959) Laboratory infection with Toxoplasma. *J. clin. Path.* 12, 59-61.

Reid, H.W. Buxton, D., Gardiner, A.C., Pow, I., Finlayson, J. & MacLean, M.J. (1982) Immunosuppression in Toxoplasmosis : Studies in Lambs and Sheep Infected with Louping-ill Virus. *J. Comp. Path.* 92, 181-190.

Remington, J.S., Krahenbuhl, J.L. & Mendenhall, J.W. (1972) A Role for Activated Macrophages in Resistance to Infection with Toxoplasma. *Infect. & Immun.* 6, 829-834.

Remington, J.S., Miller, M.J. & Brownlee, I. (1968) IgM antibodies in acute toxoplasmosis II Prevalence and significance in acquired cases. *J. Lab. clin. Med.* 71, 855-866.

Reynolds, H.Y., Kazmierowski, J.A. and Newball, H.E. (1975) Specificity of opsonic antibodies to enhance phagocytosis of Pseudomonas aeruginosa by human alveolar macrophages. *J. Clin. Invest.* 56, 376-385.

Riemann, H.P., Willadsen, C.M., Berry, L.J., Behymer, D.E. Garcia, Z.V., Franti, C.E. & Ruppanner, R. (1977) Survey for Toxoplasma antibodies among sheep in Western United States. *J. Am. vet. med. Ass.* 171, 1260-1264.

Ruiz, A. & Frenkel, J.K. (1980) Intermediate and transport hosts of Toxoplasma gondii in Costa Rica. *Am. J. Trop. Med. Hyg.* 29, 1161-1166.

Russell, O.G. and Wilhelm, H. (1986) The involvement of the major surface glycoprotein (gp 3) of Leishmania promastigotes in attachment to macrophages. 136, 1-8.

Rying, F.W. and Remington, J.S. (1977) Effect of Alveolar macrophages on Toxoplasma gondii. *Infect. Immun.* 18, 746-753.

Rytel, M.W. & Jones, T.C. (1966) Induction of Infection in mice with Toxoplasma gondii. *Proc. Soc. exp. Biol. Med.* 123, 862-866.

Sabin, A.B. (1941) Toxoplasmic encephalitis in children. *JAMA.* 116, 801-807.

Sabin, A.B. (1949) Complement fixation in Toxoplasmosis and persistence of the antibody in human beings. *Pediatrics.* 4, 443-453.

Sabin, A.B. & Feldman, H.A. (1948) Dyes As Microchemical Indicators of a New Immunity Phenomenon Affecting a Protozoan Parasite (Toxoplasma). *Science.* 108, 660-663.

Sakurai, H., Takei, Y., Omata, Y. & Suzuki, N. (1981) Production and Properties of Toxoplasma Growth Inhibitory Factor (Toxo-GIF) and Interferon (IFN) in the Lymphokines and the Circulation of Toxoplasma Immune Mice. *Zbl. Bakt. Hyg., I. Abt. Orig.* 251, 134-143.

Sanger, V.L., Chamberlain, D.M., Chamberlain, K.W., Cole, C.R. & Farrell, R.L. (1953) Toxoplasmosis V. Isolation of Toxoplasma from cattle. *J. Am. vet. med. Assoc.* 123, 87-91.

Sanger, V.L. & Cole, C.R. (1955) Toxoplasmosis VI Isolation of Toxoplasma from milk, placentas, and newborn pig of asymptomatic carrier sows. *Am. J. vet. Res.* 16, 536-539.

Schupp, E., Michel, R., Raether, W., Niemeitz, H. Uphoff, M. (1978) Invasion von Erythrozyten durch Toxoplasma. 55, 189-193.

Schreiber, R.D. & Feldman, H.A. (1980) Identification of the activator system for antibody to Toxoplasma as the classical complement pathway. *J. Infect. Dis.* 141, 366-369.

Seah, S.K.K. & Hucal, G. (1975) The use of irradiated vaccine in immunisation against experimental murine toxoplasmosis. *Canad. J. Microbiol.* 21, 1379-1385.

Sethi, K.K., Rahman, A. Pelster, B. & Brandis, H. (1977) Search for the presence of lectin-binding sites on Toxoplasma gondii. *J. Parasit.* 63, 1076-1080.

Sharma, S.D., Araujo, F.G. and Remington, J.S. (1984) Toxoplasma antigen isolated by affinity chromatography with monoclonal antibody protects mice against lethal infection with Toxoplasma gondii. *J. Immunol.* 133, 2818-2820.

Sharma, S.P. & Dubey, J.P. (1981) Quantitative survival of Toxoplasma gondii tachyzoites and bradyzoites in pepsin and in trypsin solutions. *Am. J. vet. Res.* 42, 128-130.

Sharma, S.P. & Gautam, O.P. (1978) Studies on some aspects of pathogenesis of experimental Toxoplasmosis in sheep. *Arch. Vet.* 13, 117-126.

Sharma, S.D., Mullenax, J., Araujo, F.G., Erlich, H.A. & Remington, J.S. (1983) Western blot analysis of the antigens of Toxoplasma gondii recognised by human Immunoglobulin M and Immunoglobulin G antibodies. *J. Immunol.* 131, 977-983.

Sharma, S.D., Verhoef, J. & Remington, J.S. (1984) Enhancement of human NK cell activity by subcellular components of Toxoplasma gondii. *Cellular Immunol.* 86, 317-326.

Sheffield, H.G. & Melton, M.L. (1968) The fine structure and reproduction of Toxoplasma gondii. *J. Parasit.* 54, 209-226.

Sheffield, H.G. & Melton, M.L. (1969) Toxoplasma gondii: Transmission through feces in absence of Toxocara cati eggs. *Science*. 164, 431-432.

Sheffield, H.G. & Melton, M.L. (1970) Toxoplasma gondii: The oocyst sporozoite and infection of cultured cells. *Science*. 167, 892-893.

Shirahata, T., Shimizu, K. & Suzuki, N. (1976) Effects of immune lymphocyte products and serum antibodies on the multiplication of Toxoplasma in murine peritoneal macrophages. *Z. Parasitenk* 49, 11-23.

Shirahata, T., Shimizu, K., Noda, S. Suzuki, N. (1977) Studies on Production of Biologically Active Substance which Inhibits the Intracellular Multiplication of Toxoplasma within Mouse Macrophages. *Z. Parasitenk* 53, 31-40.

Shirahata, T. & Shimizu, K. (1979) Some physiochemical characteristics of an immune lymphocyte product which inhibits the multiplication of Toxoplasma within mouse macrophages. *Microbiol. Immun.* 23, 17-30.

Shirahata, T. & Shimizu, K. (1980) Production and properties of immune interferon from spleen cell cultures of Toxoplasma infected mice. *Microbiol. Immun.* 24, 1109-1120.

Sibley, L.D., Weidner, E. & Krahenbuhl, J.L. (1985) Phagosome acidification blocked by intracellular Toxoplasma gondii. *Nature.* 315, 416-419.

Smith, I.D. (1961) Ovine Toxoplasmosis as a cause of reproductive wastage. Preliminary observations. *Aust. vet. J.* 18-21.

Smith, J.B., McIntosh, G.H. & Morris, B. (1970) The traffic of cell through tissues: a study of peripheral lymph in sheep. *J. Anat.* 107, 87-100.

Spence, J.B., Beattie, C.P., Faulkner, J., Henry, L. and Watson, W.A. (1978) Toxoplasma gondii in the semen of rams. *Vet. Res.* 102, 38-39.

Splendore, A. (1908) Un nuovo protozo parassita de conigli incontrato nelle lesioni anatomiche d'un malattiache ricorda in moltopunti il kalaazar dell'uomo nota preliminaure pel. *Rev. Soc. Sci. Sao Paulo.* 3, 109-112.

Strannegard, O. (1962) Studies of Toxoplasma precipitinogens and their corresponding antibodies by means of diffusion-in-gels methods. *Br. J. exp. Pathol.* 43, 600-613.

Stray-Pederson, B. & Lorentzen-Styr, A.M. (1980) Epidemiological aspects of Toxoplasma infections among women in Norway. *Acta. obstet. gynecol. scand.* 59, 323-

Strickland, G.T., Pettit, L.E. and Voller, A. (1973) Immunodepression in mice infected with Toxoplasma gondii. *Amer. J. Trop. Med. Hyg.* 22, 452-455.

Suzuki, Y & Kobayashi, A. (1983) Suppression of Unprimed T and B cells in Antibody Responses by Irradiation Resistant and Plastic-Adherent Suppressor cells in T.gondii Infected Mice. *Infect. Immun.* 40, 1-7.

Suzuki, Y. & Kobayashi, A. (1985a) Requirement for calcium ions in antibody-dependent complement-mediated cytolysis of Toxoplasma gondii. *Zbl. Bakt. Hyg. A.* 259, 426-431.

Suzuki, Y. & Kobayashi, A. (1985b) Suppressive effect of secondary Toxoplasma gondii infection on antibody responses in mice. *Infect. & Immun.* 48, 686-689.

Suzuki, M.Y., Tsunematsu and Torisu, M. (1971) Studies on the accessory factor for the Toxoplasma dye test: essential role of complement. *J. Parasitol.* 57, 924-925.

Suzuki, T., Watanabe, N. & Kobayashi, A. (1981a) Nonspecific Suppression Of Primary Antibody Responses and Presence of Plastic-Adherent Suppressor Cells in Toxoplasma gondii Infected Mice. *Infect. Immun.* 34, 30-35.

- Suzuki, Y., Watanabe, N., Kobayashi, A. (1981b) Nonspecific Suppression of Initiation of Memory Cells in Toxoplasma gondii Infected Mice. *Infect. Immun.* 36-42.
- Teale, A.J., Blewett, D.A., Miller, J.K. & Buxton, D. (1982) Experimentally induced toxoplasmosis in young rams : The clinical syndrome and secretion of Toxoplasma. *Vet. Rec.* 111, 53-55.
- Thorburn, H. & Williams, H. (1972) A stable haemagglutinating antigen for detecting Toxoplasma antibodies. *J. clin. Path.* 25, 762-767.
- Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and source applications. *Proc. nat. Acad. Sci. USA.* 76, 4350-4354.
- Uggla, A. Beskow, P., Schwan, O., Bergquist, N.R. & Waller, T. (1983) Ovine Toxoplasmosis in Sweden. *Acta. vet. scand.* 24, 113-119.
- Uggla, A. & Hjort, M. (1984) A serological study on the prevalence of Toxoplasma gondii in meat-producing animals in Sweden. *Acta. vet. scand.* 25, 567-576.
- Voller, A., Bidwell, D.E., Barttell, A., Fleck, D.G., Perkins, M. & Oladehin, B. (1976) A microplate enzyme-immunoassay for Toxoplasma antibody. *J. clin. Path.* 29, 150-153.
- Waldeland H. (1976a) Toxoplasmosis in sheep: the prevalence of Toxoplasma antibodies in lambs and mature sheep from different parts of Norway. *Acta. vet. scand.* 17, 432-440.
- Waldeland, H. (1976b) Toxoplasmosis in sheep - the relative importance of the infection as a cause of reproductive loss in sheep in Norway. *Acta. vet. scand.* 17, 412-425.
- Waldeland, H. (1977) Toxoplasmosis in sheep-long term epidemiological studies in four breeding flocks. *Acta. vet. scand.* 18, 227-236.
- Wadeland, H. and Frenkel, J.K. (1983) Live and killed vaccines against toxoplasmosis in mice. *J. Parasitol.* 69, 60-65
- Wallace, G.D. (1969) Isolation of Toxoplasma gondii from the feces of naturally infected cats. *J. Infect. Dis.* 124, 227-228.
- Wallace, G.D. (1973) Intermediate and transport hosts in the natural history of Toxoplasma gondii. *Amer. J. Trop. Med. & Hyg.* 22, 456-464.
- Wallace, G.D., Marshall, L. & Marshall, M. (1972) Cats, rats & toxoplasmosis on a small Pacific island. *Am. J. Epidemiol.* 95, 475-482.
- Wallace, G.D., Zigas, V. & Carleton Gajduset, D. (1974) Toxoplasmosis and cats in New Guinea. *Am. J. trop. Med. & Hyg.* 23, 8-14.

- Walton, B.D., de Arjona, I. & Brenchoff, B.M. (1966) Relationship of Toxoplasma antibodies to altitude. Am. J. trop. Med. Hyg. 15, 492-495.
- Walls, K.N., Bullock, S.L. & English, D.K. (1977) Use of the enzyme-linked immunosorbent assay (ELISA) and its microadaptation for the serodiagnosis of Toxoplasmosis. J. Clin. Microbiol. 5 273-277.
- Wanko, T., Jacobs, L. & Gavin, M.A. (1962) Electron microscope study of Toxoplasma cysts in mouse brain. J. Protozool. 9, 235-242.
- Warren, J. & Sabin, A.B. (1942) Complement fixation reaction in toxoplasmic infection. Proc. Soc. exp. biol. med. 51, 11-14.
- Watson, W.A. & Beverley, J.K.A. (1971) Ovine Abortion due to Experimental Toxoplasmosis. Vet. Rec. 88, 42-45.
- Watson, A.D.J., Farrow, B.R.H. & McDonald, P.J. (1982) Prevalence of Toxoplasma gondii antibody in pet dogs and cats. Aust. vet. J. 58, 213-214.
- Weinman, D. & Chandler, A.H. (1954) Toxoplasmosis in swine and rodents. Reciprocal oral infection and potential human hazard. Proc. Soc. exp. Biol. Med. 87, 211-216.
- Werk, R. (1985) How does Toxoplasma gondii enter host cells. Rev. Infect. Dis. 7, 4.
- Werk, R., Dunker, R. & Fischer, S. (1984) Polycationic Polypeptides: a possible model for the penetration-enhancing factor in the invasion of host cells by Toxoplasma gondii. J. Gen. Virol. 130, 927-933.
- Wickam, N. & Carne, H.R. (1950) Toxoplasmosis in domestic animals in Australia. Aust. vet. J. 26, 1-3.
- Wilson, B.M. and Nakane, P.K. (1978) In immunofluorescence and related staining techniques. Ed. by W. Knapp. 215-224.
- Wilson, C.B., Tsai, V. & Remington, J.S. (1980) Failure to trigger the oxidative metabolic burst by normal macrophages. Possible mechanism for survival of intracellular pathogens. J. exp. Med. 151, 328-346.
- Williams, K. & Williams, H. (1979) Toxoplasmosis in Scotland 1974-7. Br. Med. J. 1, 561.
- Wolf, A. & Cowen, D. (1937) Granulomatous encephalomyelitis due to an encephalitozoon (encephalitozic encephalomyelitis). A new protozoan disease of man. Bull. Neurol. Inst. New York. 6, 306-335.
- Work, K. (1968) Resistance of Toxoplasma gondii encysted in pork. Acta. pathol. microbiol. scand. 73, 85-92.
- Yano, K. & Nakabayashi, T. (1980) Immunological analyses of antigens associated with experimental toxoplasmosis. Biken's J, 23, 33-41.

APPENDIX

Publication relating to this thesis

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STUDIES ON OVINE EFFERENT LYMPH FOLLOWING INFECTION WITH TOXOPLASMA GONDII

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INTRODUCTION

Toxoplasma gondii, a protozoan parasite with a worldwide distribution infects most species of warm blooded animals. It was first recognised as a cause of ovine abortion and neonatal loss by Hartley and Marshall in New Zealand (1957) and the first cases in the U.K. were reported in 1961 (Beverley and Watson).

The immune response to T.gondii in man and mice involves both humoral and cellular mechanisms (Hoff and Frenkel, 1974). Anti-toxoplasma antibodies enhance phagocytosis of tachyzoites (Jones, Len and Hirsch, 1975) and if antibody is present before tachyzoites enter the cell, intracellular multiplication can be decreased (Shirahata, Shimizu, and Suzuki, 1976). Thymus - derived lymphocytes enhance the killing of T.gondii by macrophages (Borges and Johnson 1975; Sakurai, Takei, Omata and Suzuki, 1981) and are required for the induction of the humoral response to the parasite (Lindberg and Frenkel, 1977, Buxton, 1980).

In sheep, humoral immunity is induced following infection with

T.gondii (Hartley, 1964, Beverley and Watson, 1971; Blewett, Bryson and Miller, 1983), although little is known about the nature of any cellular response that might take place. Lymphocytes infiltrate tissues infected with T.gondii (Koestner and Cole 1961; Sharma and Gautam 1978) and recent work by Buxton and Finlayson (1986) has suggested that a cellular immune response can also occur in foetuses following infection in utero.

The local immune response of sheep to subcutaneous infection with T.gondii has been studied and the changes in the infected lymph node resemble a Toxoplasma-induced lymphadenopathy as seen in man, rabbits and mice (Buxton, Miller, Finlayson and Wallace, 1981). Furthermore, with the technique of lymphatic cannulation (Buxton et al, 1981), live toxoplasms were detected in the lymph of non-immune sheep throughout the experiment, but lymph from immune animals challenged with T.gondii did not contain live organisms.

In the present study lymphatic cannulation was used to monitor cell types in lymph after primary and secondary infection with T.gondii.

MATERIALS AND METHODS

Inoculum

T.gondii (M1 strain) originally isolated from an aborted ovine foetus was grown in mice (passage 8 and 9) and inoculum prepared from their brains as previously described (Buxton, Reid and Pow, 1979). Control inoculum was prepared in the same manner from the brains of normal uninfected mice.

Animals

Cheviot, Merino and Scottish Blackface breeds of sheep were employed. One popliteal efferent lymphatic duct of each of fourteen animals was cannulated according to published procedures (Hall and Morris, 1962). Twelve of the sheep had no anti-toxoplasma antibody detectable by enzyme linked immunosorbent assay (ELISA) (Voller, Bidwell, Bartlett, Fleck, Perkins and Oladehin, 1976; Buxton and Finlayson, 1986.) while the remaining two sheep had naturally acquired antibody. Seven of the susceptible animals and the two with naturally acquired antibody (groups I and II, respectively) were each given 100 T.gondii tissue cysts by subcutaneous inoculation on the lateral aspect of the cannulated leg below the hock joint. The remaining five (group III) were injected with control inoculum in the same manner.

Clinical Observations

Sheep were examined daily for clinical signs of illness, including a febrile response.

Cell Output

Lymph was sampled at least twice daily and the flow rate calculated. Cell numbers were counted with a model ZBI Coulter counter (Coulter Electronics Ltd, Luton, England) fitted with a 100µm aperture tube. The instrument was set to count particles greater than 45 µm.

Cell Types

Lymph cells were washed three times in Hanks' balanced salt solution (HBSS) and the concentration adjusted to 1×10^6 per ml in one per cent fetal calf serum in HBSS. Cytocentrifuge preparations (Shandon Ltd, Runcorn, England) were made with 200 μ l samples of this suspension. One preparation was stained by Leishman's solution and a differential cell count performed. The remaining preparations were fixed for ten minutes in two per cent paraformaldehyde, washed with phosphate buffered saline (PBS), and stored in Tris saline buffer.

Immunoglobulin-containing cells

Antisera against sheep IgM and IgG were prepared as previously described (Buxton and Finlayson, 1986). Briefly, antiserum to sheep IgM was raised in a rabbit, the IgG fraction was extracted and rendered monospecific by affinity chromatography. Antiserum against sheep IgG was raised in a pig and the IgG fraction prepared by ion exchange chromatography. Both fractions had their anti-light chain activity removed by affinity chromatography, and were then conjugated with horseradish peroxidase (Sigma type V1) (Wilson and Nakane, 1978). Cytocentrifuge preparations were removed from Tris saline buffer and incubated with either of the above conjugated antisera for 90 minutes. Histochemical colouration of the enzyme was achieved by the application of 3'-3 diaminobenzidine (4mg in 10ml 0.05M Tris HCl buffer containing 0.01 per cent hydrogen peroxide).

Anti-toxoplasma antibody in lymph

The presence of specific IgG anti-toxoplasma antibody was measured by ELISA (Buxton and Finlayson, 1986).

Necropsy

Sheep were killed by intravenous injection of pentobarbitone sodium (300mg) after the lymph had ceased flowing. The popliteal and internal iliac lymph nodes were removed from both sides and small blocks of tissue were selected and fixed in modified Bouin, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax (Finlayson, Anderson, Buxton and Donald, 1985). Cells containing IgM and IgG were stained by an immunoperoxidase technique as previously described (Buxton and Finlayson, 1986). Further material from these lymph nodes was fixed in Baker's calcium formol and processed to paraffin wax.

RESULTS

Clinical Observations

A temperature greater than 40°C was detected in all susceptible sheep infected with T.gondii by day 5 or 6 after infection and lasted for an average of 5 days. No other clinical signs were observed. Both the immune and control sheep (groups II and III, respectively) remained clinically normal.

Cell output from the popliteal efferent lymph duct

Following inoculation with T.gondii tissue cysts, sheep in group I (naive challenge) showed a steady rise in total cell output (Fig 1) which reached a peak on day 6 that was sustained until

day 10 and by day 16 it had returned to the mean preinoculation value. The total cell output of animals in immune challenge group II rose until day 5 when it was twice the value of the preinoculation counts. The average number of cells in the efferent lymph from uninfected control sheep (Group III) reached a peak by day 5 when it showed a threefold increase over preinoculation numbers.

The proportion of lymphoblast cells, defined as large cells with irregular nuclei and basophilic cytoplasm, present in the lymph of sheep in group I increased throughout the course of infection (Fig 2). By the peak response on day 11, lymphoblasts accounted for 50.3 per cent of the total cell output while in contrast, the response in group III reached a peak of 13.9 per cent on day 8. It was shown by Student's t-test that the lymphoblast responses in group I on days 10 and 11 were significantly different ($p < 0.02$ and $p < 0.05$, respectively) from those in the control sheep. The peak of the lymphoblast cell response in group II (27.5 per cent) occurred on day 4.

After inoculation with T.gondii, the percentage of lymphocytes containing IgM or IgG rose in both group I and II (Figs 3a and 3b). In group I, both responses reached their peaks on day 7 when a total of 12 per cent of all cells contained Ig. By day four, 11.5 per cent of all lymphocytes from sheep in group II contained IgM whereas the IgG response reached a peak one day later when 10.5 per cent of the cells were positively stained. Lymph from control sheep showed a slight increase in positive cells, but the total number of cells that contained Ig never exceeded 4 per cent on any one day.

Antibody

Specific IgG was first detected on day 9 in ewes that received a primary infection (group I) whilst the amount in the immune animals (group II) rose daily (Fig 4). No anti-toxoplasma antibody was detected in control animals in group III at any time.

Necropsy

Efferent lymph flowed for a mean period of 12 days in group I, 10 days in group II and 9 days in the controls. In group I, popliteal and internal iliac lymph nodes from the cannulated and injected side of the animals were enlarged and measured between 25 and 30mm long and 20mm wide. The contralateral lymph nodes were similar in size to those in control animals and measured 10 to 15 mm by 7.5mm. The lymph nodes from animals in group II animals were intermediate in size.

Histopathology

Changes in the infected lymph nodes were the same as previously described (Buxton et al, 1981).

DISCUSSION

The local immune response in sheep to both primary and secondary infection with T.gondii tissue cysts was monitored by cannulation of the popliteal efferent lymphatics. As already reported, only sheep experiencing a primary infection showed a febrile response (Miller, Blewett and Buxton, 1982; Blewett, Miller and Buxton, 1982). Although a large proportion of lymphoblasts was present in the efferent lymph of these sheep,

few of them contained either IgM or IgG. Specific anti-toxoplasma IgG antibody was detected by day 9 while the peak of the lymphoblast response occurred after the peak of the Ig-containing cell response.

From these findings, it appears that during the primary response to infection with T.gondii the majority of the activated cells do not contain Ig. Although some of these Ig negative cells may be null cells (Fahey 1980; Outteridge, Fahey and Lee, 1981) it seems likely that most of them are T cells, as at the time of this study no better method of identifying ovine T cells was available. Histological examination of the draining lymph node in this and a previous study (Buxton et al, 1981) demonstrated expansion of both the cortex and the medulla indicating that the parasite evoked both B and T cell responses. It is therefore interesting that it is predominantly T cells which are found in the efferent lymph, while B cells appear to be confined to the lymph node. T cells have been shown to have a functional role in the immune response to T.gondii in other species, including man and mice and it is possible that they also play a role in the immune response of sheep to T.gondii. In vitro, in the presence of T.gondii, primed T cells can secrete certain factors, which include Toxoplasma growth inhibition factor (Sakurai et al, 1981) and gamma interferon (Shirahata and Shimizu, 1980). These lymphokines stimulate macrophages, resulting in increased intracellular killing of Toxoplasma (Sakurai et al, 1981). Murine recombinant gamma interferon can also have a significant activity against T.gondii in vivo since its presence enhances the antibody response to the parasite

(McCabe, Luft and Remington, 1984). These lymphokines also regulate both oxygen-dependent and oxygen-independent antimicrobial killing by macrophages (Murray, Byrne, Rothermel and Cartelli, 1983). It is apparent, therefore, that they play a central role in the immune response to T.gondii; whether they do so in sheep has yet to be established.

When animals naturally immune to T.gondii were challenged with the parasite, the cellular composition of the efferent lymph differed from that of the animals experiencing a primary infection. The lymphoblast response was also less dramatic but more rapid in onset and its peak coincided with the peak of the Ig cell response, when more than 50 per cent of cells contained either IgM or IgG. Following primary infection with T.gondii, efferent lymph from the infected node can contain live organisms, while lymph from immune animals does not (Buxton et al, 1981). What specific immune mechanisms are involved is uncertain, but one possibility is that antibody which is present at the time of a challenge infection, opsonises the parasite, leading to increased phagocytosis and intracellular killing by macrophages, as has been reported in other species (Jones et al, 1975, Shirahata et al, 1976). This mechanism could not occur as rapidly in a primary infection, since IgG antibody is not detected until day 9 allowing the multiplication of T.gondii to proceed unhindered in the initial stages of infection. The serological test used in this study did not detect IgM antibody and therefore the presence of IgM in lymph soon after infection cannot be excluded. However, IgM antibody to Toxoplasma has not been detected before day 10 in the sera of experimentally

infected sheep (Blewett et al, 1983), so it is unlikely to be present in the lymph immediately after infection.

While this paper gives the first indication of the cell types activated following infection of sheep with T.gondii, further work is required to establish the roles played by antibody-producing cells and, particularly, T cells, in protecting the host from this parasite.

SUMMARY

Cell output was monitored in efferent popliteal lymphatics of sheep following subcutaneous injection into the lateral tarsus of either one hundred T.gondii tissue cysts or control inoculum. Sheep undergoing a primary infection developed a substantially increased lymphoblast output which reached a peak of more than 50 per cent of all cells. The majority of lymphoblasts did not contain Ig and may have been T cells. IgG antibody against T.gondii was detected in lymph by day 9. Animals with naturally acquired antibody to T.gondii developed a more rapid lymphoblast response to the parasite which, at its peak, accounted for 27.5 per cent of all cells. Many of these lymphoblasts contained Ig, presumptively specific antibody, which, by increasing phagocytosis and intracellular killing of the T.gondii, would prevent multiplication and dissemination of the parasite.

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REFERENCES

Beverley J.K.A. and Watson W.A. (1961). Ovine abortion and toxoplasmosis in Yorkshire. *Veterinary Record*, 73, 6-10.

Beverley J.K.A. and Watson W.A. (1971). Prevention of experimental and of naturally occurring ovine abortion due to toxoplasmosis. *Veterinary Record*, 88, 39-41.

Blewett D.A., Bryson C.A. and Miller J.K. (1983). Studies of antibody titres in experimentally induced ovine toxoplasmosis. *Research in Veterinary Science*, 43, 163-166.

Blewett D.A., Miller J.K. and Buxton D. (1982). Response of immune and susceptible ewes to infection with Toxoplasma gondii. *Veterinary Record*, 111, 175-177.

Borges J. and Johnson W (1975). Inhibition of multiplication of T.gondii by human monocytes exposed to T lymphocyte products. *Journal of Experimental Medicine*, 141, 483-496.

Buxton D. (1980). Experimental infection of athymic mice with Toxoplasma gondii. *Journal of Medical Microbiology*, 13, 307-311.

Buxton D. and Finlayson J. (1986). Experimental infection of pregnant sheep with Toxoplasma gondii : pathological and immunological observations on the placenta and foetus. *Journal of Comparative Pathology*, 96, 319-333.

Buxton D., Miller H.R.P. Finlayson J. and Wallace G.R. (1981). Toxoplasma gondii - its effect on the ovine popliteal lymph node. Journal of Medical Microbiology, 14, 435-442.

Buxton D., Reid H.W. and Pow I. (1979). Immunosuppression in toxoplasmosis, studies in mice with a clostridial vaccine and Louping-ill virus vaccine. Journal of Comparative Pathology, 89, 375-379.

Fahey K.J. (1980). The binding of lectins to sheep tissue and circulation cells: peanut agglutinin, a marker for presumptive T lymphocytes. Australian Journal of Experimental Biology and Medical Research, 58, 557-569.

Finlayson J., Anderson I., Buxton D. and Donald, K.M. (1985). A direct immunoperoxidase method for demonstrating Chlamydia psittaci in tissue sections. Journal of Clinical Pathology, 38, 712-714.

Frenkel J.K. (1973). in The Coccidia, edited by D.M. Hammond and P.L.Long, University Park Press, Baltimore, p343-410.

Hall J.G. and Morris B (1962). The output of cells in the lymph from the popliteal node of sheep. Queensland Journal of Experimental Physiology, 47, 360-368.

Hartley J.K.A. (1964). Experimental transmission of toxoplasma in ewes showing high and low dye test titres. New Zealand Veterinary Journal, 12, 6-8.

Hartley J.K.A. and Marshall S.C. (1957). Toxoplasmosis as a cause of ovine perinatal mortality. New Zealand Veterinary Journal, 5, 119-124.

Hoff R.L. and Frenkel J.K. (1974). Cell-mediated immunity against Besnoitia and Toxoplasma in specifically and cross-immunised hamsters and in cultures. Journal of Experimental Medicine, 139, 560-580.

Jones T.C., Len L. and Hirsch J.G. (1975). Assessment in vitro of immunity against Toxoplasma gondii. Immunology, 141, 466-482.

Koestner A. and Cole C.R. (1961). Neuropathology of ovine and bovine toxoplasmosis. American Journal of Veterinary Research, 22, 55-56.

Lindberg R.E. and Frenkel J.K. (1977). Toxoplasmosis in nude mice. Journal of Parasitology, 63, 219-221.

McCabe R.E., Luft B.J. and Remington J.S. (1984). Effect of murine interferon gamma on murine toxoplasmosis. Journal of Infectious Diseases, 150, 961-922.

Miller J.K., Blewett D.A. and Buxton D. (1982). Clinical and serological response of pregnant gimmers to experimentally induced toxoplasmosis. *Veterinary Record*, 111, 124-126.

Murray H.N., Byrne G.I., Rothermel C.D. and Cartelli D.M. (1983). Lymphokine enhances oxygen-independent activity against intracellular pathogens. *Journal of Experimental Medicine* 158, 234-239.

Outteridge P.M., Fahey K.J. and Lee C.S. (1981). Lymphocyte surface markers in sheep blood and lymph. *Australian Journal of Experimental Biology and Medical Research*, 59, 143-155.

Sakurai H., Takei Y., Omata Y. and Suzuki N. (1981). Production and growth inhibitory factor (Toxo-GIF) and interferon (IFN). *Zentralblatt fur Bakteriologie Parasitenkunde Infektionskrankheiten und Hygiene*, 1, 124-143.

Sharma S.P. and Gautam O.P. (1978). Studies on some aspects of pathogenesis of experimental toxoplasmosis in sheep. *Archiva Veterinaria*, 13, 117-126.

Shirahata T. and Shimizu K. (1980). Production and properties of immune interferon from spleen cell cultures of Toxoplasma infected mice. *Microbiology Immunology*, 24, 1109-1120.

Shirahata T., Shimizu K. and Suzuki N. (1976). Effects of immune lymphocyte products and serum antibody on the multiplication of Toxoplasma in murine peritoneal macrophages. *Zeitschrift fur Parasitenkunde*, 49, 11-23.

Wilson B.M. and Nakane P.K. (1978). Recent developments in the periodate method of conjugating horse radish peroxidase (HPR) to antibodies. In Immunofluorescence and related staining techniques. Edited by Knapp W. Holubar K. Wick G. Elsevier/North Holland Biomedical Press 215-224.

Voller A., Bidwell D.E., Bartlett A., Fleck D.G., Perkins M. and Oladehin B. (1976). A microplate enzyme-immunoassay for toxoplasma antibody. Journal of Clinical Pathology, 29, 150-153.

LEGENDS

Fig 1 The mean cell output from the cannulated efferent lymphatic ducts of sheep inoculated with one hundred T.gondii tissue cysts; susceptible sheep — immune sheep — — or control inoculum — — — .

Fig 2 The mean percentage of lymphoblast output from the cannulated efferent lymphatic ducts of sheep inoculated with one hundred T.gondii tissue cysts; susceptible sheep — immune sheep — — or control inoculum — — — .

Fig 3 a) The mean number of IgM-containing cells in the efferent lymph of sheep inoculated with T.gondii tissue cysts; susceptible sheep — immune sheep — — or control inoculum — — — .

Fig 3b) The mean number of IgG-containing cells in the efferent lymph of sheep inoculated with T.gondii tissue cysts; susceptible sheep — immune sheep — — or control inoculum — — — .

Figure 4 Antitoxoplasma IgG in the efferent lymph of susceptible (—) or immune (— — —) sheep following inoculation with T.gondii tissue cysts, or in susceptible sheep after control inoculum — — — .

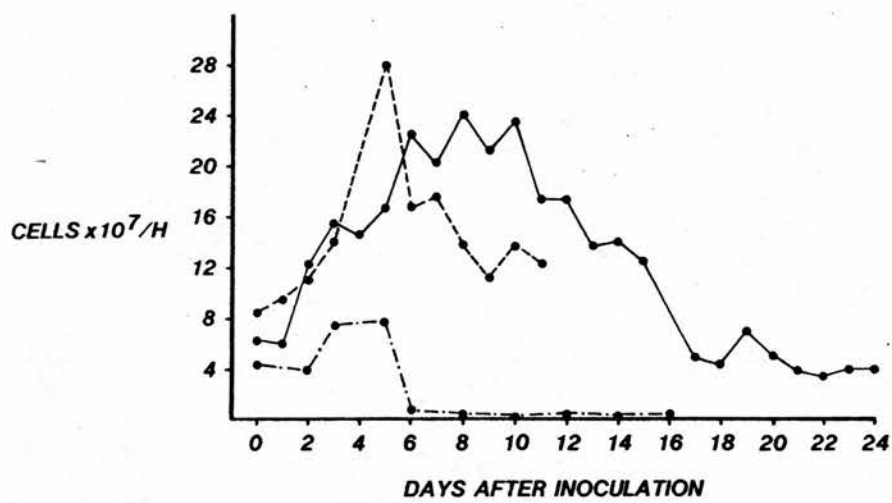


Fig 1

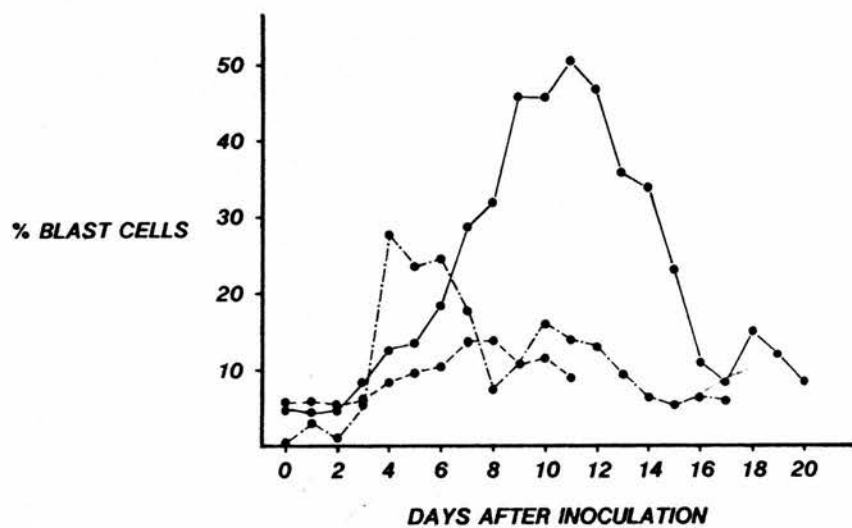


Fig 2

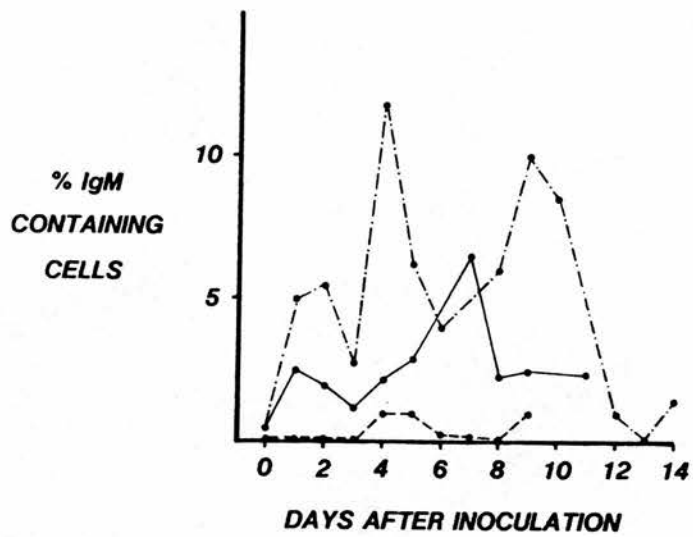


Fig 3a

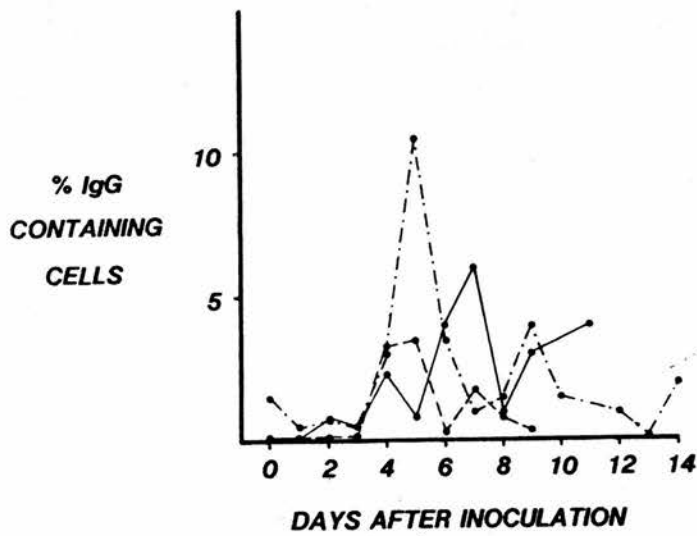


Fig 3b

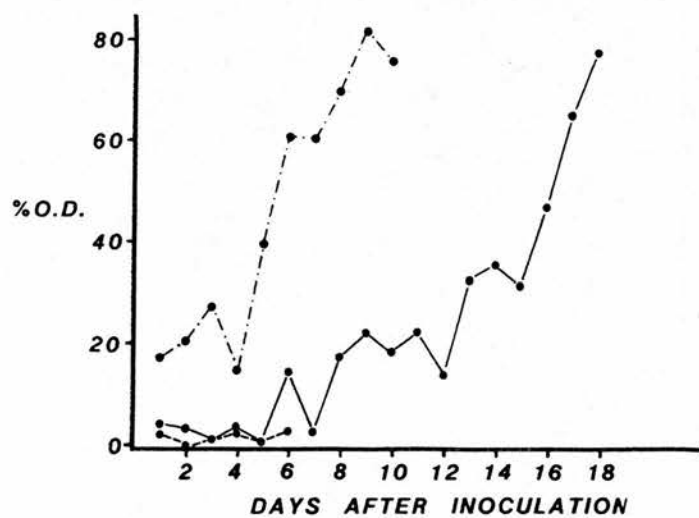


Fig 4